



# EFFECTOR/MEMORY CD8 T CELL DYNAMICS IN HIV-INFECTED PATIENTS DURING HAART DISCONTINUATION AND RESUMPTION

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## BACKGROUND AND RATIONALE

- Highly Active Antiretroviral Therapy (HAART) resulted in dramatic changes in the disease course, but long-term therapy is limited by the need of strict daily-adherence, occurrence of drug-toxicity, elevated treatment-costs and sustained risks for the emergence of drug-resistance.
- Structured Treatment Interruption (STI) may help to reduce the patient time for receiving drugs. However, the data to justify STI as a safe and effective approach remain controversial.
- HIV-specific CD8<sup>+</sup> T lymphocytes are important in the antiviral response. However, these CTL fail to completely control the infection. A skewed maturation of HIV-specific CTL has been recently demonstrated, indicating that the HIV-specific CTL pool is largely composed by pre-terminally differentiated CCR7<sup>-</sup> CD45RA<sup>-</sup> CD8<sup>+</sup> T lymphocytes with poor cytotoxic activities.
- CCR7<sup>-</sup> effector T-cells have lost the ability for autoregulation, display specialized function such as IFN- $\gamma$  production and an acquire mature cytolytic function after CD45RA expression. T-cell activation induces a transient increase of CD27 expression that gradually switches off on effector cells.
- Monitoring both CCR7 and CD27 surface expression allows one to discriminate among different effector or long-term memory T-cell subsets.

## AIM

Aim of the study was to analyze the influence of STI on effector/memory dynamics of CD8<sup>+</sup> T-cell subsets in chronically HIV-infected patients showing a rapid or delayed viral rebound. CD8<sup>+</sup> T-cell reactivity to mitogen and HIV-Gag peptides was also analyzed.

## PATIENTS

Asymptomatic chronically HIV-1 infected patients were recruited from the National Institute for Infectious Diseases "Lazzaro Spallanzani". The criteria for inclusion in the study were:

- HAART (at least 2 years of successful virus suppression),
- stable CD4<sup>+</sup> T-cell counts above 500/mm<sup>3</sup> for at least 12 months before entry,
- undetectable HIV RNA plasma levels (< 50 copies/ml by branched DNA) for at least 12 months before entry,
- HAART discontinuation for side effects or spontaneous request.

All selected patients underwent a single-cycle STI consisting of at least 1 month of discontinuation. HAART re-introduction was decided according to the guidelines on antiretroviral therapy (CD4<sup>+</sup> T-cells < 350/mm<sup>3</sup> and/or HIV-RNA > 30,000 copies/ml). Twenty-six patients completed the study protocol. Two groups were selected on the basis of plasma viral rebound. Group A (n=14) included patients with a rapid viral rebound (within 1 months) and Group B (n=6) was composed of patients with a delayed viral rebound (after 4 months) (Table 1). Six patients presenting an intermediate behavior were not considered. Clinical and immunological follow-up in Group A was performed at the time of HAART suspension (t0), after 1 month from the suspension (t1), at the resumption of HAART (t2), and after 30 days from HAART-resumption (t3). Group B patients were monitored at HAART suspension (t0) and after every month during suspension (t1a, t1b, t1c, etc.).

All patients gave written informed consent and the protocol was approved by the Institute's Ethical Committee.

## Main characteristics of HIV-infected patients undergoing STI

### Group A. Rapid viral rebound (HIV-RNA > 30,000 copies/mL)

Patient	Sex	Age	HAART	Cause of discontinuation	CD4	Viral rebound (days)
BG21	m	48	3tc-d4t-nfv	lipodystrophy	623	26
FR04	m	47	d4t-3tc-nvp	lipodystrophy	788	27
FN24	f	54	azt-3tc-idx	lipodystrophy	1097	27
MC25	f	44	3tc-ddi-idx	lipodystrophy	528	27
GG29	m	47	3tc-d4t-nfv	patient's request	862	28
RR36	m	41	ddi-d4t-rvt	hypertriglyceridemia	1014	28
MB03	f	36	d4t-3tc-efv	lipodystrophy	795	28
DV13	f	38	d4t-3tc-idx	patient's request	512	28
FG11	f	31	d4t-3tc-nvp	patient's request	625	28
SA31	f	32	azt-3tc-rvt	patient's request	567	28
CU06	m	47	azt-3tc-rvt	patient's request	1195	28
VM08	f	50	azt-3tc-nfv	patient's request	501	28
DA18	m	55	3tc-d4t-nvp	lipodystrophy	632	28
BM23	m	38	3tc-d4t-efv	lipodystrophy	581	34

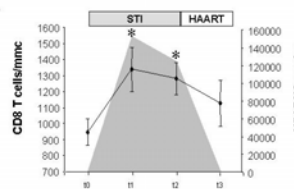
### Group B. Delayed viral rebound (HIV-RNA < 30,000 copies/mL)

Patient	Sex	Age	HAART	Cause of discontinuation	CD4	Viral rebound (days)
MC10	f	40	d4t-3tc-efv	lipodystrophy	502	144
MA30	m	43	azt-3tc-idx	nephrolithiasis	636	150
FR33	m	31	azt-3tc-idx	patient's request	1718	184
LM28*	m	34	azt-3tc	lipodystrophy	673	no
HL05*	f	26	azt-ddi-idx	lipodystrophy	759	no
SA01*	f	37	d4t-3tc	lipodystrophy	878	no

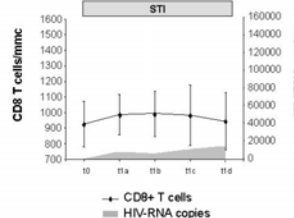
\*These patients are still without HAART after 359, 634 and 736 days of suspension, respectively

## Immunological and virological parameters of the HIV-STI study groups

### Group A



### Group B



CD8 T lymphocytes/mm<sup>3</sup> and plasma viremia were analyzed in Group A patients (n=14) and in patients with a delayed viral rebound (Group B, n=6). CD8 T-cell count is presented along the left ordinate. HIV-1 plasma RNA copies are presented by the gray area along the right ordinate. The absolute number of CD8 T-cells are expressed as mean values  $\pm$  standard error. Plasma HIV-RNA was performed by Quantiplex HIV-1 RNA version 3.0 assay (bDNA v3.0; Bayer Diagnostics, Emeryville, CA) according to manufacturer's specifications. \*, p<0.01 respect to t0 and t3

## METHODS

### Selection of Gag HIV-1 epitope peptides.

28 different 15-mers containing one or more epitopes putatively able to bind to at least two different HLA-serological specificities for each HLA class I locus (A, B or C) and recognised by HLA class I gene products from two different loci were selected on the consensus sequences of the most conserved areas of HIV-1 Gag protein. The selected peptides were synthesized from Sigma-Gensys (Cambridge, UK) as free aminoacids. All synthetic peptides were purified by reverse-phase chromatography (RPC) to >90% purity. Sequence and purity were confirmed by mass spectrometry and analytical RPC.

### Monoclonal antibodies and flow cytometry

The anti-human monoclonal antibodies (mAbs) coupled with fluorescein (FITC), phycoerythrin (PE), phycoerythrin-cyanin 5.1 (PE-Cy5) and allophycocyanin combined for simultaneous staining used in this study were: anti-CD4, anti-CD8, anti-CD27, anti-CD45RA, anti-IFN- $\gamma$  mAb and control-mAb (IgG1). The purified anti-CCR7 was detected using biotin-conjugated rat anti-mouse IgM and streptavidin PE. All mAbs were obtained from Becton Dickinson, Mountain View, CA. Flow cytometric analysis was performed on a FACSscan flow cytometer (Becton Dickinson). At least 100,000 live events were acquired, gated on small viable lymphocytes. Data files were analyzed using CellQuest software (Becton Dickinson).

### Cell isolation and stimulation

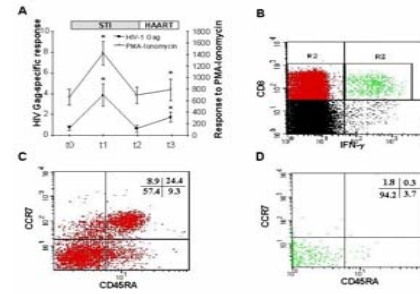
Peripheral blood mononuclear cells (PBMC) were obtained using standard Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density centrifugation and frozen in DMSO at -80°C. Briefly, 1x10<sup>8</sup> thawed cells in 1 ml of complete RPMI 1640, 10% v/v heat-inactivated FCS, 2 mM L-Glutamine, 10 U/ml penicillin/streptomycin, were incubated with 1  $\mu$ g each of anti-CD28 and CD49d mAbs and pooled Gag-peptides (1  $\mu$ g of each peptide) or with PMA (50ng/ml) and ionomycin (10 $\mu$ g/ml). Cells incubated with only anti-CD28 and CD49d were included in every experiment as control samples. The cultures were finally incubated at 37°C in a 5% CO<sub>2</sub> incubator for 1 h, followed by an additional 5 hr of incubation in the presence of 10  $\mu$ g/ml of Brefeldin-A to inhibit cellular exocytosis Sigma, St. Louis, MO.

### Statistical analysis

Differences among groups were evaluated by Wilcoxon's paired test.

## CD8<sup>+</sup> T-cell dynamics during STI in rapid viral rebound group (1)

HIV-specific IFN- $\gamma$  producing CD8<sup>+</sup> T-cells are expanded during STI and mainly expressed the CD45RA<sup>-</sup> CCR7<sup>-</sup> phenotype of pre-terminally differentiated effector cells

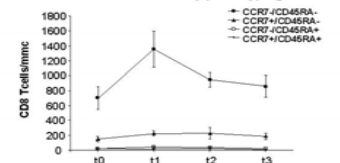


IFN- $\gamma$  producing CD8<sup>+</sup> T-lymphocytes were analyzed after HIV-specific (Gag-peptides, left ordinate) and mitogenic (PMA-Ionomycin, right ordinate) stimulation (Panel A). A FACS dot-plot of CD8<sup>+</sup> T-cell response after Gag peptide stimulation from a representative HIV-infected patient during STI (t1, DA18) is shown in Panel B. After HIV-Gag stimulation, CD8<sup>+</sup> T-cells unresponsive (R2) or IFN- $\gamma$  producing (R3) were monitored for CCR7 and CD45RA expression (Panel C, and D respectively). The frequency of naive/effector/memory CD8<sup>+</sup> T-cells is shown on gate R2 (CD8<sup>+</sup> IFN- $\gamma$ +, Panel C) and on gate R3 (CD8<sup>+</sup> IFN- $\gamma$ +, Panel D). The absolute number of CD8<sup>+</sup> T-cells is expressed as mean values  $\pm$  standard error. \*, p<0.01 in comparison with t0

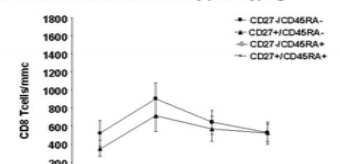
## CD8<sup>+</sup> T-cell subset dynamics during STI in rapid viral rebound group (2)

The general expansion of CD8<sup>+</sup> T-cell after STI is largely composed by immature CD8<sup>+</sup> expressing the CD45RA<sup>-</sup> CCR7<sup>-</sup> phenotype (A). Almost half of these T-cells also expressed the CD27 molecules (B), supporting a block at specific stages of CD8<sup>+</sup> T-cell differentiation including CD27<sup>+</sup> CD45RA<sup>-</sup> long-term memory cells.

### A. CCR7/CD45RA effector/memory phenotyping



### B. CD27/CD45RA effector/memory phenotyping



## CONCLUSIONS

An accumulation of pre-terminally differentiated CCR7<sup>-</sup> CD27<sup>+/−</sup> CD45RA<sup>-</sup> CD8<sup>+</sup> T-cells was associated to the viral rebound during STI, demonstrating that failure to control viremia is related to the accumulation of CD8<sup>+</sup> T-cells at a specific stage of differentiation.

Most STI patients experiencing a rapid viral rebound have sustained HIV specific CD8<sup>+</sup> T-cells producing IFN- $\gamma$ , but the majority of these cells present a phenotype that has been shown not to be functionally cytotoxic, leaving the patient with high numbers of non-functional virus-specific CD8<sup>+</sup> T-cells.

This impaired CTL function and accumulation of pre-terminally differentiated CD8<sup>+</sup> T-cells may be a consequence of an increased turnover of terminally differentiated CD8<sup>+</sup> T-cells or a lack of antigen specific CD4<sup>+</sup> T-cell helper activity.

Adjunct therapies, such as the use of immune modulators, could be directed to restore CTL effector functions improving the quality of the antiviral immune response.