

# Early reduction of immune activation in lymphoid tissue following highly active HIV therapy

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**Objective:** To evaluate immune reconstitution within HIV-infected lymphoid tissue during highly active antiretroviral therapy (HAART).

**Design and methods:** *In situ* cellular responses were studied in sequential tonsillar biopsies in three asymptomatic HIV-infected (CD4 cells greater than  $400 \times 10^6/l$ ) antiretroviral treatment-naïve volunteers enrolled in a clinical trial to determine the early effect of HAART. Computerized image analysis was used to study immunohistochemically stained sequential tonsil sections for the patterns of local cytokine production, chemokine receptor expression and cellular distribution. Replicate quantitative assessments of samples before and after 4 weeks of therapy were used for the evaluation of drug effects and compared with four uninfected controls. Tonsillar HIV proviral-DNA was determined by fluorescent *in situ* 5'-nuclease assay.

**Results:** HIV-infected tonsil tissue was characterized by extensive pro-inflammatory and type 1 cytokine expression. A five- to 15-fold elevation of interleukin (IL)-1 $\alpha$ , IL-12, IL-2 and interferon (IFN)- $\gamma$  protein expression was found compared with controls, and each encompassed a mean of at least 4.5% of the tissue compartment. This was reduced by 20–90% in all individuals after 4 weeks of HAART. In contrast, type 2 cytokine expression (IL-4, IL-10), plus tumour necrosis factor (TNF)- $\alpha$ , remained low throughout the study. HAART reduced, by 40%, the expression of HIV co-receptors, CCR5 and CXCR4, which initially were elevated four to six times over the control values. In addition, the myelomonocytic inflammatory proteins, CD68 and calprotectin, diminished by 26–83% after therapy. The HIV RNA was reduced to undetectable levels in plasma by HAART. However, a large pool of tonsil cells (2–7%), remained HIV DNA positive after 4 weeks of therapy.

**Conclusions:** Although immune activation may be the direct consequence of HIV replication, HAART-associated reconstitution begins with a reduction in inflammatory cytokine production which precedes the elimination of local proviral reservoirs.

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**Keywords:** Cytokines, highly active antiretroviral therapy, immunopathogenesis, lymphoid tissue, HIV coreceptors, viral load, image analysis

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## Introduction

A complex relationship exists between the immune system and HIV within peripheral lymphoid tissue [1,2]. Highly active antiretroviral therapy (HAART) has resulted in a significant reduction of the HIV plasma load and partial restoration of peripheral CD4 cell function and counts [3,4], while the knowledge of the effects of HAART on immune restoration within lymphoid tissue is hitherto more restricted [5]. Immune impairment may persist, even after long term therapy [6] and result in viral recurrence even after 30 months of HAART [7].

In order to elucidate the complex interactions between HIV replication and the immune system we evaluated tonsil biopsies in asymptomatic HIV-positive patients prior to and after 4 weeks of HAART. Tonsils can contain a 10- to 1000-fold higher HIV RNA load than plasma and thus provide a resource for studying the effects of antiviral therapy [8,9]. This provided an opportunity to evaluate the direct effects imposed by local HIV replication on the immune system, and *de novo* immune activity manifested after abrogation of HIV replication. The hypothesis tested here was that HIV replication induces an 'immune syndrome' which facilitates viral persistence. We quantified the HAART-mediated changes in immune activity by measuring the expression of local cytokines, chemokine receptors and cell activation in conjunction with viral load.

## Materials and methods

### Patients and controls

Three HIV-seropositive asymptomatic antiretroviral treatment-naïve individuals (Centers for Disease Control stage A1) with CD4+ blood counts greater than  $400 \times 10^6$  cells/l blood were recruited for this study from the clinical trial Intercompany Study 004. Institutional review board approval was obtained from Rush Medical School. The patients were biopsied prior to and 4 weeks after therapy with zidovudine, lamivudine (3TC) and indinavir. Tonsil biopsies from four adult seronegative controls were obtained after institutional review board approval from Huddinge University Hospital.

### Evaluation of cell free and in-cell HIV viral load

HIV RNA measurements in plasma were performed in all patients with the Amplicor assay (Roche, Nutley, New Jersey, USA) with the lower limit of detection of 400 HIV RNA copies/ml.

### HIV DNA containing cells detected by fluorescent *in situ* 5'-nuclease assay

Tonsillar tissue was fixed overnight in molecular biology grade Streck Tissue Fixative (MBF) and paraf-

fin-embedded, sectioned and deparafinized, before amplification with a HIV-1 *gag* fluorogenic probe and imaged [10].

### Detection of cytokines, chemokine receptors and cellular markers by immunohistochemistry

Tonsil cryostat sections, 8-10  $\mu\text{m}$  thick, were fixed and permeabilized as previously described [11]. The specificity of the primary monoclonal antibody (MAb) used to detect interleukin (IL)-1 $\alpha$ , IL-1 $\beta$ , tumour necrosis factor (TNF)- $\alpha$ , IL-12 (p40 and p70), IL-2, interferon (IFN)- $\gamma$ , IL-4 and IL-10 (PharMingen, San Diego, California, USA) was controlled by the abolishment of immunoreactivity by preabsorption of the MAb with the corresponding cytokine protein. Cytokine-cDNA transfected eukaryotic cells expressing the target cytokine protein were used for positive control. Quantitative assessments of the staining for cytokines and CCR5 (M:45549.11 and K:45531.111, mouse IgG2b; R&D Systems, Minneapolis, Minnesota, USA), CXCR4 (44717.111, mouse IgG2b; R&D Systems), CD1a (mouse MAb; Immunocytometry Systems, San Jose, California, USA), HLA-DR (mouse IgG2b MAb; Becton Dickinson, San Jose, California, USA), calprotectin and CD68 (Dakopatts, Glostrup, Denmark) was performed by computerized image analysis

### Acquired computerized image analysis quantification of cytokines, chemokine receptors and phenotypes of cells

Images were transferred from a DMR-X microscope (Leica, Wetzlar, Germany), to a computerized image analyser (Quantimet Q550IW; Leica, Cambridge, UK), allowing separation of 16.7 million different colours. The total tissue ( $1-8 \times 10^6 \mu\text{m}^2$ ) was assessed for positive-stained cells which were counted for all cells ( $3.2-25.0 \times 10^5$ ) present within the total tissue area, in a semiquantitative way using a specialized software program [12,13]. Cytokine-expressing cells could be identified at the single cell level due to the characteristic staining pattern of protein accumulated in the Golgi-stacks [12]. Limiting dilution of cDNA transfected cells injected to the tonsils indicated a sensitivity of the assay of at least 1 positive cell/1000 events [12]. Each parameter was analysed in triplicate.

## Results

### Effect of HAART on HIV burden in peripheral blood and tonsils

A significant reduction in plasma HIV RNA ( $1-3 \log_{10}$ ) was observed in the patients to below detectable levels after 4 weeks of HAART (Table 1). The incidence of HIV-1 provirus DNA containing cells, determined by fluorescent *in situ* 5'-nuclease assay, decreased less dramatically in tonsil tissue ( $0.3-0.7 \log_{10}$ ), and a large

**Table 1.** Effect of highly active antiretroviral therapy on HIV-1 viral load and T-lymphocyte subsets.

Therapy	Study subject					
	1		2		3	
	Before	After	Before	After	Before	After
Sample						
Peripheral blood						
HIV-1 RNA (copies/ml)	6780	< 400	3770	< 400	23250	< 400
T-lymphocyte						
CD4%	36	46	36	44	43	40
CD8%	48	44	24	25	47	32
CD4 absolute $\times 10^6/l$	403	529	878	598	374	460
CD8 absolute $\times 10^6/l$	538	506	586	340	409	368
CD4 : CD8*	0.8	1.05	1.49	1.75	0.91	1.25
Tonsil tissue						
HIV-1 DNA% cells <sup>†</sup>	3.4	2.7	8.2	7.8	7.2	2.1

\*Derived from absolute lymphocyte counts. <sup>†</sup>Fluorescent *in situ* 5'-nuclease assay.

pool of HIV DNA positive cells (2.1–7.8% of tonsillar cells) continued to be maintained (Fig. 1).

### HIV co-receptor expression (CCR5 and CXCR4) is influenced by HAART

Acquired computerized image analysis was used to compare immune markers in tonsils from HIV-positive patients and HIV-negative controls (Fig. 2). The CD8+ T cells, CCR5 and CXCR4 expressing cells were increased four- to eightfold in untreated HIV-infected patients as compared with controls (Fig. 3). The expression of the HIV co-receptors CCR5 and CXCR4 were down-regulated following HAART (to 19–42% below baseline; Fig. 3).

### Sequestering of CD8+ cells in HIV-positive tonsils

The initial levels of peripheral blood CD4+ cells ( $403\text{--}878 \times 10^6/l$ ) and CD8+ cells ( $489\text{--}586 \times 10^6/l$ ) in our HIV-positive patients before therapy were only moderately affected after HAART (Table 1). As observed in other studies, peripheral CD4+ cells tended to increase while CD8+ cells were delayed at this early time point. Thus, the CD4+ : CD8+ ratio in blood was uniformly higher following therapy. In comparison with controls, the tonsils of HIV-positive subjects showed five- to eightfold higher levels of CD8+ cells, in distributions which were predominantly extrafollicular. HAART therapy for 4 weeks did not significantly diminish either these levels of tonsil CD8+ cells nor significantly alter the proportion of CD4+ cells in the tonsil (Fig 3).

### Expression of type 1 and type 2 immune cytokines within tonsils

A 10- to 15-fold increase in IL-2 and IFN- $\gamma$  expressing cells was found compared with the control tissue (Fig. 3), and in contrast to controls, type 1 cytokines predominated over type 2 responses by 10- to 20-fold both before and after antiviral therapy (Fig 3). A profound IFN- $\gamma$  expression, 5–15% of cellular compartment, was observed prior to therapy and reduced

by HAART to 28–77% of baseline (Fig. 3). The increased IL-2 level in tonsils before treatment (0.7–6.3% of cellular compartment) was also reduced by therapy (to 12–87% of baseline; Fig. 3). The IL-4 expression represented 0.4–1.4% of the cellular compartment and exceeded the IL-10 expression (0.01–0.7% of cellular content) within the tonsil (Fig. 3).

### Reduction in pro-inflammatory cytokine production by HAART

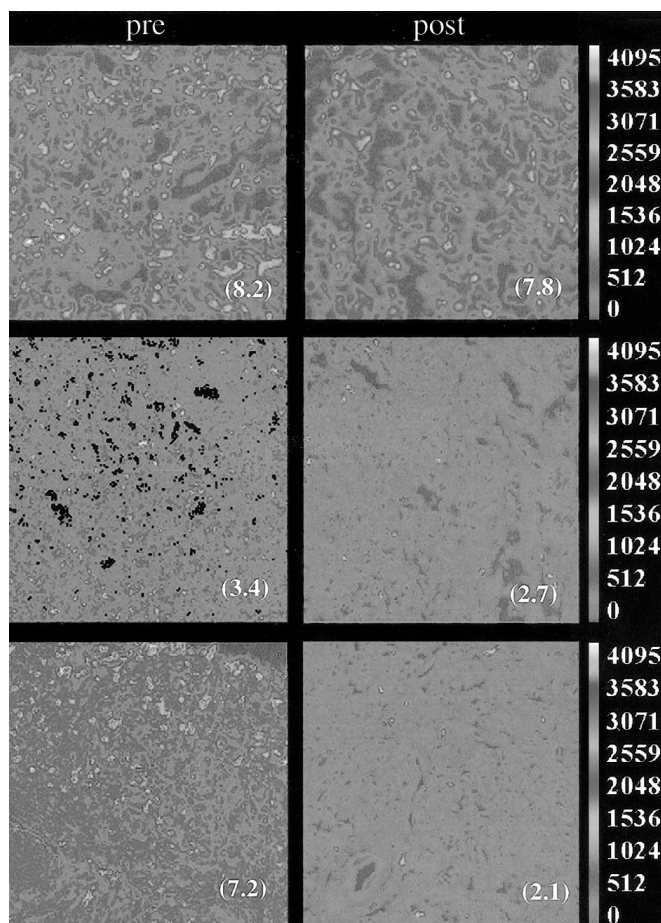
HIV-infected tonsil tissue was characterized by a four- to 12-fold increased expression of IL-1 $\alpha$ , IL-1 $\beta$ , IL-12, and TNF- $\alpha$  (Fig. 4). Untreated HIV-1-infected patients showed high prevalence of IL-1 $\alpha$ , IL-1 $\beta$  and IL-12 containing cells encompassing 1–8% of all cells (Fig. 4). A significant reduction to less than 50% of pretreatment levels was observed following 4 weeks of HAART (Fig. 4). Minor changes were noticed for TNF- $\alpha$  after institution of HAART.

### Activation of myelomonocytic cells is reduced by HAART

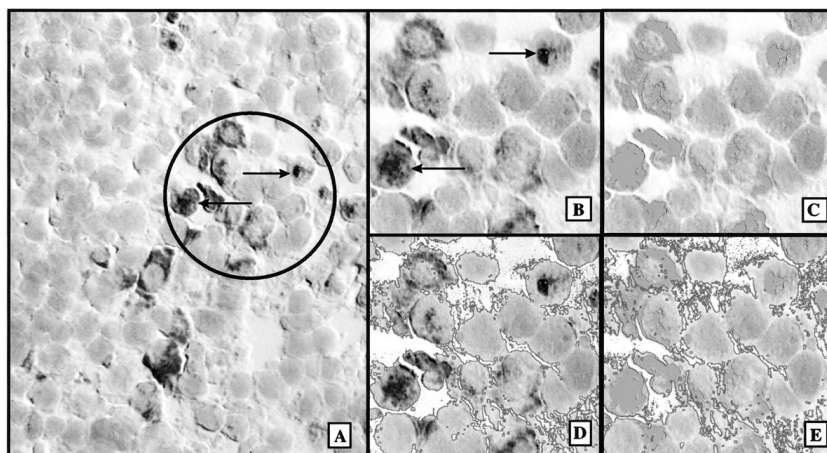
We measured three myeloid antigens, CD68, calprotectin and CD1a as indices of macrophage and dendritic cell activation and HLA-DR expression (Fig. 4). Two- to threefold higher levels than in controls were observed in all HIV-1-positive patients prior to therapy. A reduction of calprotectin, CD68, CD1a and HLA-DR levels were noticed in all patients by HAART (Fig. 4).

## Discussion

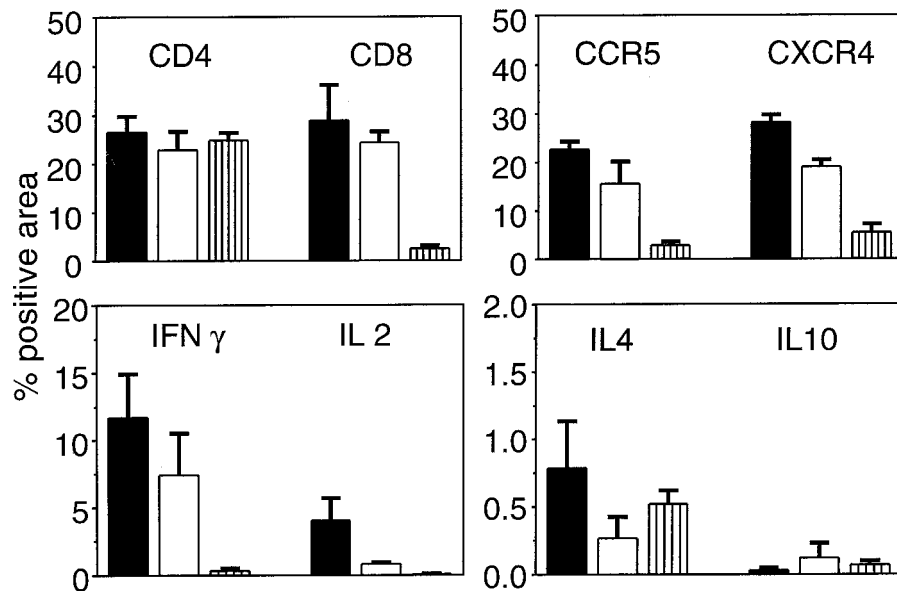
Convincing data demonstrate that lymphoid tissue represents a major reservoir of HIV replication [3,8]. It has been suggested that the lymphoid microenvironment is advantageous for HIV multiplication due to the activation of target cells and the production of acces-



**Fig. 1.** Detection of HIV DNA by fluorescent *in situ* 5'-nuclease assay in tonsils from three HIV-infected patients. (a-c) Cells containing HIV DNA, blue to red (mean fluorescence intensity, 2815). Uninfected cells, purple-black (mean fluorescence intensity, 256). Left panel, samples obtained prior, and right panel, after highly active antiretroviral therapy. Percentage HIV-1 proviral DNA-containing cells in whole biopsies are in parenthesis.



**Fig. 2.** (A) Digital images-illustrating interleukin (IL)-2 from an HIV-infected tonsil biopsy expressing cells (brown) counter-stained with hematoxylin (blue). (→), Lymphocytes expressing IL-2 in a juxta-nuclear pattern associated with the Golgi complex. A detailed area, encircled in micrograph A is shown in B-E. Individual picture elements (pixel) defining the brown RGB colour thresholds of the IL-2 staining, were calibrated for size in relationship to the magnification (for B-E, 1 pixel = 0.118  $\mu$ m), and marked in yellow by acquired computerized image analysis (C). The area occupied by hematoxylin counter-staining was measured in a similar way (D, red contour lines). A super-imposed overlay of each of these detected features (IL-2 in yellow, total cells in red) is outlined on the original micrograph (E). In the presented microscopic field the IL-2 positive stained area comprised 4.2% of the total cellular compartment.



**Fig. 3.** The effect of highly active antiretroviral therapy (HAART) on the frequency of CD4, CD8, CCR5 and CXCR4-expressing cell subsets in tonsil. The black bars represent patients before, and white bars after, 4 weeks of HAART. The striped bars represent controls. Assessments by acquired computerized image analysis of CD4, CD8, chemokine receptor expression and of type 1 [interferon- $\gamma$ , interleukin (IL)-2] and type 2 (IL-4, IL-10) cytokines are indicated.

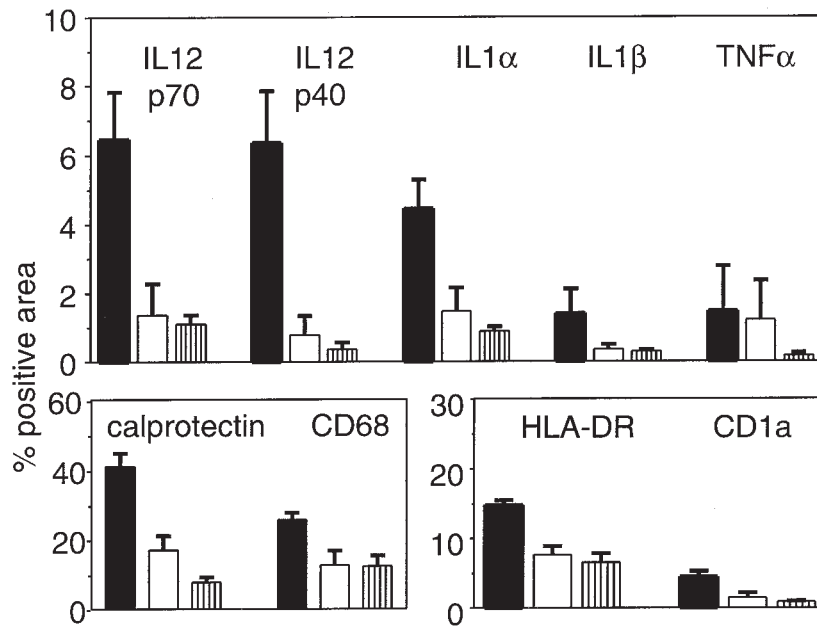
sory growth factors [3,5,]. Therefore, this process of mobilization and activation of immune competent cells supported by local cytokine activity paradoxically may contribute to the propagation of virus replication. Plasma and mRNA cytokine measurements have provided evidence for increased production of IL-1 $\beta$ , TNF- $\alpha$ , IL-6, IL-8 and IFN- $\gamma$  even in asymptomatic HIV-infected patients while IL-2, IL-4 and IL-10 have been below detectable levels [14–16]. Evaluation of the significance of plasma or whole blood cytokine levels within the frame of HIV immunopathogenesis of HIV is complicated within tissue, due to the fact that cytokine production is compartmentalized. Serum concentrations of these cytokines, and CCR5 binding chemokines, RANTES, macrophage inflammatory protein (MIP)-1 $\alpha$  and MIP-1 $\beta$ , have not been shown to be of prognostic value [17]. But there is evidence for increased mRNA content of IL-1 $\beta$ , IL-4, IL-6, IL-10 in addition to IL-2 and IFN- $\gamma$  mRNA positivity in the lymph nodes of HIV-positive patients [1,18]. These activities are exhausted with extended duration of disease, characterized by a decline of IL-2, IL-12 and IFN- $\gamma$  production in combination with an increase of IL-4, IL-5, IL-6 and IL-10 [19].

We evaluated immune activation as well as systemic and local viral load by applying novel approaches that combined immunocytochemistry and quantitative image analysis. Profound evidence for HIV-associated immune activation was found in tonsil tissue which included an upregulated expression of the calprotectin, CD68, CD1a expression and HLA-DR. In addition, extensive IL-1 $\alpha$ , IL-1 $\beta$ , IL-12, IL-2 and IFN- $\gamma$  expres-

sion occurred in different regions within the tonsillar biopsies (Figs 3, 4). The IL-12, IFN- $\gamma$  and IL-2 production was approximately 10- to 15-fold higher than that found in control tissue. Others have reported excessive tissue and peripheral blood mononuclear cells (PBMC) mRNA expression of IFN- $\gamma$  and IL-10 whereas IL-2 and IL-4 were barely detectable regardless of disease state [18].

*In vitro* experiments indicate that cell surface expression of CCR5 and CXCR4 chemokine receptor expression are under cytokine regulation [20]. Both CXCR4 and CCR5 receptor expression were elevated before therapy in the HIV-infected tonsils and reduced following therapy, in parallel with the reduction in immune activation. Thus, within tissue we observed an association between local increases and drops of cytokine expression and regulation of chemokine receptor expression.

HAART may reduce the plasma viral load of HIV-1-infected individuals to undetectable levels [21]. However, the effects of these regimens are on latently infected CD4+ T cells and what potential these cells play in the persistence of HIV-1 infection in individuals receiving HAART are unclear. Proviral DNA capable of producing infectious virus upon cellular activation *in vitro*, have been found in such patients. In addition, HIV-1 DNA in infected resting CD4+ T cells from patients with undetectable plasma viremia after 6 months of HAART, also suggest continual persistent active virus replication.



**Fig. 4.** Highly active antiretroviral therapy (HAART) diminution of pro-inflammatory cytokine expression [interleukin (IL)-1 $\alpha$ , IL-1 $\beta$ , tumour necrosis factor (TNF)- $\alpha$ , IL-12] and cell activation (calprotectin, CD68, CD1a and HLA-DR) are expressed as percentage of positive cells out of total tonsillar tissue area as determined by acquired computerized image analysis. Black bars, before HAART; white bars, 4 weeks of HAART in HIV patients, and striped bars, control tonsils.

Evidence from this pilot study of tissue before and after HAART therapy would imply that it is actually the HIV replication that induces the monokine and type 1 cytokine synthesis at the local site. As such, the pool of residual HIV within tissue is both regulating immune activity and dependent upon immune activation.

HAART induced impressive down regulation of immune activation within tissue prior to a significant drop of HIV proviral DNA expression. This effect may have been caused by diminished homing and retention of T-cells to the tissue compartment due to drops in local chemokine production. Or alternatively, caused by a reduction in proliferation of activated cells within tissue. There are findings supporting both explanations including (i) the initial biphasic increase of CD45 RO+ (memory) T-cells followed by a slow CD45RA+ (naive) T-cell gain in peripheral blood T-cell after initiation of HAART, which favours the first hypothesis [22] or (ii) a reduction in activation markers such as HLA-DR, CD38 and Ki-67 which argues for the latter mechanism [23,24]. We feel that it is possible that both chains of events contribute to the observed immune reconstitution. In essence, a complete picture of these events cannot be formed until we can define the fate of individual cells using combined indices of proliferation, apoptosis, migration and influx. Indeed, the preliminary observations in this current study need to be confirmed by extended investigations.

## References

1. Gray CM, Morris L, Murray J, et al.: **Identification of cell subsets expressing intracytoplasmic cytokines within HIV-1-infected lymph nodes.** *AIDS* 1996, **10**:1467-1475.
2. Scarlatti G, Tresoldi E, Bjorndal A, et al.: **HIV escapes chemokine control, changes coreceptor usage in parallel.** *Nature Med* 1997, **3**:1259-1265.
3. Chun TW, Carruth L, Finzi D, et al.: **Quantification of latent tissue reservoirs, total body viral load in HIV-1 infection.** *Nature* 1997, **387**:183-188.
4. Ho DD, Neumann AU, Perelson AS, Chen W, Leonard JM, Markowitz M: **Rapid turnover of plasma virions, CD4 lymphocytes in HIV-1 infection.** *Nature* 1995, **373**:123-126.
5. Cohen OJ, Pantaleo G, Lam GK, Fauci AS: **Studies on lymphoid tissue from HIV-infected individuals: implications for the design of therapeutic strategies.** *Springer Semin Immunopathol* 1997, **18**:305-322.
6. Connors M, Kovacs JA, Krevat S, et al.: **HIV infection induces changes in CD4+ T-cell phenotype, depletions within the CD4+ T-cell repertoire that are not immediately restored by antiviral or immune-based therapies.** *Nature Med* 1997, **3**:533-540.
7. Finzi D, Hermankova M, Pierson T, et al.: **Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy.** *Science* 1997, **278**:1295-1300.
8. Haase AT, Henry K, Zupancic M, et al.: **Quantitative image analysis of HIV-1 infection in lymphoid tissue.** *Science* 1996, **274**:985-989.
9. Cavert W, Notermans DW, Staskus K, et al.: **Kinetics of response in lymphoid tissues to antiretroviral therapy of HIV-1.** *Science* 1997, **276**:960-964.
10. Patterson BK, Jiyamapa D, Mayrand E, et al.: **Detection of HIV-1 DNA in cells, tissue by fluorescent *in situ* 5'-nuclease assay (FISNA).** *Nucleic Acids Res* 1996, **24**:3656-3658.
11. Andersson J, Abrams J, Björk L, et al.: **Concomitant *in vivo* production of 19 different cytokines in human tonsils.** *Immunology* 1994, **83**:16-24.

12. Björk L, Fehniger T, Andersson U, Andersson J: **Computerized assessment of production of multiple human cytokines at the single-cell level using image analysis.** *J Leukocyte Biol* 1996, **59**:287–295.
13. Litton M, Dohlsten M, Rosendahl A, et al.: **The functional significance of a collaboration between CD4+, CD8+ T cells in generating a successful cytotoxic anti-tumor attack.** *Am J Pathol* 1997, **150**:1607–1618.
14. Salazar-Gonzalez JF, Martinez-Maza O, Aziz N, et al.: **Relationship of plasma HIV-RNA levels, levels of TNF-alpha, immune activation products in HIV infection.** *Clin Immunol Immunopathol* 1997, **84**:36–45.
15. Than S, Hu R, Oyaizu N, et al.: **Cytokine pattern in relation to disease progression in human immunodeficiency virus-infected children.** *J Infect Dis* 1997, **175**:47–56.
16. Navikas V, Link J, Persson C, et al.: **Increased mRNA expression of IL-6, IL-10, TNF-alpha, perforin in blood mononuclear cells in human HIV infection.** *AIDS Res Hum Retrovirus* 1995, **15**:484–489.
17. McKenzie SW, Dallalio G, North M, Frame P, Means Jr RT: **Serum chemokine levels in patients with non-progressing HIV infection.** *AIDS* 1996, **10**:F29–F33.
18. Graziosi C, Pantaleo G, Gantt KR, et al.: **Lack of evidence for the dichotomy of TH1, TH2 predominance in HIV-infected individuals.** *Science* 1994, **265**:248–252.
19. Shearer GM, Clerici M: **Protective immunity against HIV infection: has nature done the experiment for us?** *Immunol Today* 1996, **17**:21–24.
20. Loetscher P, Ugucioni M, Bordoli L, et al.: **CCR5 is characteristic of Th1 lymphocytes.** *Nature* 1998, **391**:344–345.
21. Perelson AS, Essunger P, Cao Y, et al.: **Decay characteristics of HIV-1-infected compartments during combination therapy.** *Nature* 1997, **387**:188–191.
22. Pakker NG, Noremans DW, Boer RJ, et al.: **Biphasic kinetics of peripheral blood T cells after triple combination therapy in HIV-1 infection: a composite of redistribution and proliferation.** *Nature Med* 1998, **4**:208–214.
23. Autran B, Carcelain G, Li TS, et al.: **Positive effects of combined antiretroviral therapy on CD4+ T cell homeostasis and function in advanced HIV disease.** *Science* 1997, **277**:112–116.
24. Tenner-Racs K, Stellbrink H-J, van Lunzen J, et al.: **The unenlarged lymph nodes of HIV-1-infected, asymptomatic patients with high CD4 T-cell counts are sites for virus replication and CD4 cell proliferation. The impact of highly active antiretroviral therapy.** *J Exp Med* 1998, **187**:949–959.