

# Effect of Antiretroviral Therapy on Apoptosis Markers and Morphology in Peripheral Lymph Nodes of HIV-Infected Individuals

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## Abstract

**Background:** CD4+ T cell depletion and destruction and the involution of the lymphoid tissue are hallmarks of HIV infection. Although the underlying mechanisms are still unclear, apoptosis appears to play a central role. The objective of this study was to investigate the effect of antiretroviral therapy on the lymph node tissue, particularly with respect to morphology and apoptosis.

**Patients and Methods:** Between 1997 and 1999, two inguinal lymph nodes were excised from 31 previously untreated individuals who were in an early stage of HIV infection, the first one prior to treatment and the second after 16 to 20 months of treatment. Paraffin sections were investigated for lymph node architecture, distribution of cellular and viral markers, apoptosis, and expression of apoptotic key molecules which indirectly reflect apoptotic processes.

**Results:** After 16–20 months of antiretroviral therapy, a significant decrease in highly activated HIV-driven immune response was observed in the lymph node tissue as a marked reduction in follicular hyperplasia, a normalization of the follicular dendritic cell network, a significant increase in the number of CD4+ T cells, and a significant decrease in the number of CD8+ T cells. The expression of several pro-apoptotic (Fas, TRAIL, and active caspase 3) and anti-apoptotic (Bcl-2 and IL-7R $\alpha$ ) molecules that were reconstituted in the tissues during therapy resembled their expression in lymph nodes of HIV-negative individuals. Limitations of the study are (a) the lack of untreated patients in the late stages, (b) for ethical reasons, the lack of a control group with untreated patients, and (c) for methodological reasons, the restriction of sequential measurements of apoptotic markers to one-third of the patients.

**Conclusion:** Antiretroviral therapy initiated in the early stages in HIV infection may halt the irreversible destruction of the lymph node tissue and may partially normalize apoptotic processes

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## Introduction

Potent antiretroviral therapy in HIV-infected patients leads to a suppression of the HIV load and to a con-

comitant increase in the number of CD4+ T cells [1–3] in the peripheral blood. However, the regulatory processes that are important for immune recovery take place in the lymphoid tissue which acts as a viral reservoir and major site of HIV replication. HIV preferentially replicates in CD4+ T lymphocytes, and virus production is 5 to 10 times higher in the lymphoid tissue than that in the peripheral blood lymphocytes [4–6]. Therefore, we compared lymph nodes that were excised from 31 previously untreated HIV-infected patients before therapy and after 16–20 months of antiretroviral therapy with respect to lymph node architecture, the distribution of cellular and viral markers, and the expression of apoptotic key molecules.

The persistence of HIV replication leads to chronic immune stimulation [7] and to typical alterations in the morphology of the lymphoid tissue [8]. Early in HIV-infection, follicular hyperplasia develops and germinal centers become infiltrated by cytotoxic CD8+ T cells. This overactive, massive immune reaction leads to a progressive destruction of follicular dendritic cells and their network within the germinal centers [9, 10]. This and the parallel depletion of CD4+ T cells lead to hypoplasia and the involution of the lymphoid tissue, resulting in a breakdown of the immune system in the advanced stage of HIV infection [11].

The underlying pathogenic mechanisms that are responsible for CD4+ T cell depletion in untreated HIV

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infection are not completely understood [12–14]. During early infection, the cell destruction involves primarily CCR5+ effector memory T cells and occurs predominantly in the mucosal tissue, particularly in the intestinal immune system [15, 16]. Disordered apoptosis and an increased rate of activation-induced cell death (AICD) lead to destabilization and progressive change of the homeostasis of resting naive and memory T cell populations [14, 17–20]. It is unclear, and even controversial, which of pro-apoptotic (e.g. Fas, Fas-ligand, TRAIL, and TRAIL-receptors) and anti-apoptotic (e.g. Bcl-2, c-Flip, and IL-7R $\alpha$ ) molecules are involved and to what extent.

## Patients and Methods

### Study Design

Between 1997 and 1999, 31 previously untreated, adult HIV-1-infected patients, 27 men and 4 women, with a mean age of  $35 \pm 10$  years were enrolled in this study. Twenty-four patients were treated with dual combination therapy that included one nucleoside reverse transcriptase and one protease inhibitor; seven patients were treated with a triple combination therapy that included one nucleoside reverse transcriptase and two protease inhibitors.

In all 31 patients, one inguinal lymph node was excised before the onset of therapy and a second node was excised after 16–20 months of therapy. The lymphoid tissue was fixed in formalin, embedded in paraffin and grouped according to the classification of the European Study Group [21]. At the time of each excision, peripheral blood was also drawn from the patient to obtain CD4+ and CD8+ T cell counts. The viral load was determined in plasma (detection limit = 500 copies/ml) by using the HIV Amplicor Monitor (Roche Switzerland).

The project was approved by the Ethical Committee of the University Hospital Lausanne. The written informed consent was obtained from all participants.

### Immunostaining of the Lymph Node Tissue

Paraffin sections of the lymph node tissue were immunohistochemically stained for the following antigens: CD4 (Novocastra, clone 1F6, dilution 1:20), CD8 (Dako Cytomation Copenhagen Denmark, clone, C8/144B), CD21 (Cell Marque, clone 2G9), p24 (Dako, clone Kal-1,1:4), Bcl-2 (Dako, clone 124), IL-7R $\alpha$  (Santa Cruz Biotechnologies, 1:100), c-Flip [22] (1:400), Fas (Novocastra, clone GM30; 1:20), FasL [22] (1:80), TRAIL (Santa Cruz Biotechnologies, K-18, 1:75), and active caspase 3 (R&D; 1:1,500). The sections were stained according to the manufacturers' instructions; horse radish peroxidase (HRP)-conjugate (Envision + system, Dako) and AEC substrate (Dako) were used except for the CD4 stains, in which the ABC-Elite-Complex (Vector laboratories Inc, Burlingame, CA, USA) and DAB substrate (Dako) were used.

Apoptotic cells were detected by using the TUNEL technique with the *In Situ* Cell Detection Kit (Roche Diagnostics) according to the manufacturer's protocol. Control slides, which were incubated only with the conjugates, did not show any staining.

The sections were counterstained with Mayer's hematoxylin (Fluka, Buchs, Switzerland) and then mounted with crystal mount (Biomedica Corp., Foster City, CA, USA).

Lymph nodes that had been excised from five HIV-negative people were used as controls for the immunohistological stains.

### Morphometry and Quantification

Morphometric measurements prior to and following therapy were focused on the secondary follicles because these best reflect the stage of HIV infection. Photographs were taken by using a Zeiss Axioplan 2 microscope (Carl Zeiss AG, Switzerland) that was equipped with a Hamamatsu camera and then analyzed with the KS300 image analysis software (Carl Zeiss AG, Switzerland). Regions of interest were segmented and the number of pixels/segment was converted into mm<sup>2</sup> by using defined areas of a Neubauer counting chamber as the reference.

The total active lymph node area (the cortex – including the germinal centers, the paracortex, and the medullary cords, but without medullary sinus and cicatrice tissue) was determined. The number and size of germinal centers were quantified per total active lymph node area on sections that were stained for Bcl-2.

The infiltration of germinal centers by CD8+ T cells was evaluated on immunohistochemically stained sections by sequentially measuring the area of up to 10 germinal centers. The proportion of stained CD8+ cells as determined by their hue, luminescence, and saturation (HLS) was taken as the grade of infiltration by CD8+ T lymphocytes.

For p24, the staining was graded only as positive or negative because the variation between follicles was too high for statistical analysis.

To quantify apoptosis in lymph node slides, the number of apoptotic cells within all visible germinal centers using the TUNEL technique was counted independently by two persons.

To quantify TRAIL, regions of interest were selected and in two to four of such regions which were converted into mm<sup>2</sup>, the number of TRAIL-positive cells was counted independently by two persons.

For all other cellular (CD4, interfollicular CD8, CD21) and apoptosis-related antigens (Bcl-2, c-Flip, IL-7R $\alpha$ , Fas, FasL, active caspase 3), the gray level intensity was determined by computer-assisted image analysis using the Openlab program (Improvision, Coventry, UK) as described by *Bachmann et al.* [22].

### Statistical Analysis

Data were analyzed for differences before and after therapy by using StatView (StatView, 1998, Abacus Concepts Inc., Berkeley, CA, USA). Wilcoxon's rank sum test was used for analysis of differences between paired observations for nonparametric distribution, and paired Student's t-test was used for differences in normally distributed variables. Results were considered to be significant when  $p < 0.05$ . Values were expressed as the mean or median together with the standard deviation (SD) or range.

## Results

### Peripheral Blood Measurements

The viral load decreased significantly ( $p < 0.0001$ ) in all 31 patients after a mean time of 18 months of therapy (Table 1) and was below the detection limit in 28 of 31 cases. The median CD4+ T cell count in all patients increased significantly (Table 1). The overall median CD8+ T cell count did not change significantly under therapy (Table 1).

### Lymph Node Histology

The majority of lymph nodes were larger before therapy than after therapy (Table 1). Twenty biopsies showed

Table 1 Alterations in peripheral blood values, lymph node architecture, and apoptosis markers under ART in patients with follicular hyperplasia (with or without fragmentation; FH) or follicular involution/depletion (F-Inv/Depl).					
	Before ART		16–20 m of ART		p-value
	n	Baseline values at first LN excision	n	Values at second LN excision	
<i>Peripheral blood</i>					
HIV-1 RNA (lg <sub>10</sub> copies/ml)	31	4.6 ± 0.45	31	2.8 ± 0.16	< 0.0001
FH	20	4.5 ± 0.49	20	2.7 ± 0.15	< 0.0001
F-Inv/Depl	11	4.6 ± 0.35	11	2.8 ± 0.19	< 0.0001
CD4+ T cells (cells/μl)	31	667 (349–1,213)	31	1,011 (278–2,458)	0.0004
FH	20	625 (349–1,088)	20	986 (278–2,458)	0.0068
F-Inv/Depl	11	742 (403–1,213)	11	1,055 (441–1,900)	0.03
CD8+ T cells (cells/μl)	31	1,162 (452–3,154)	31	1,004 (275–2,826)	NS
FH	20	1,171 (463–3,154)	20	1,086 (275–2,826)	NS
F-Inv/Depl	11	1,147 (452–1,775)	11	1,240 (349–2,340)	NS
<i>Lymph node architecture</i>					
Reactive LN area (mm <sup>2</sup> )/section	31	19.5 ± 7	31	12.1 ± 6	< 0.0001
FH	20	19.4 ± 7	9	13.8 ± 8	0.002
F-Inv/Depl	11	19.7 ± 5	22	11.4 ± 6	0.003
Mean GC size (mm <sup>2</sup> )	31	1.8 ± 2	31	0.28 ± 0.7	0.0003
FH	20	2.7 ± 2	9	0.7 ± 1.2	< 0.0001
F-Inv/Depl	11	0.26 ± 0.3	22	0.11 ± 0.15	NS
GC number/section	31	12 ± 8	31	6 ± 5	0.0004
FH	20	16 ± 7	9	11 ± 4	0.0004
F-Inv/Depl	11	5 ± 6	22	4 ± 4	NS
CD8+T infiltration of GC (mm <sup>2</sup> )	26	0.013 ± 0.01	18	0.001 ± 0.003	< 0.0001
FH	19	0.015 ± 0.01	7	0.003 ± 0.005	0.0015
F-Inv/Depl	7	0.006 ± 0.01	11	0.001 ± 0.001	NS
<i>Markers in lymph nodes</i>					
CD4 (interfollicular) <sup>a</sup>	15	1,738 ± 166	15	1,939 ± 143	< 0.0001
FH	9	1,749 ± 189	2	2,101 ± 30	0.008
F-Inv/Depl	6	1,722 ± 139	13	1,914 ± 137	0.03
CD8 (interfollicular) <sup>a</sup>	16	1,736 ± 88	16	1,516 ± 81	< 0.0001
FH	11	1,726 ± 83	3	1,474 ± 47	0.003
F-Inv/Depl	5	1,759 ± 105	13	1,526 ± 85	0.04
CD21 (follicular) <sup>a</sup>	12	845 ± 110	12	1,017 ± 100	0.008
FH	10	868 ± 103	6	988 ± 75	0.02
F-Inv/Depl	2	727 ± 61	6	1,047 ± 120	NS
Bcl-2 (mantle zone) <sup>a</sup>	13	1,218 ± 142	13	1,480 ± 198	< 0.0001
FH	10	1,217 ± 132	6	1,504 ± 242	0.005
F-Inv/Depl	3	1,218 ± 207	7	1,460 ± 169	NS
IL-7R $\alpha$ (follicular) <sup>a</sup>	8	875 ± 128	8	947 ± 89	0.01
FH	8	875 ± 128	6	965 ± 91	0.01
F-Inv/Depl	0		2	892 ± 80	
TRAIL <sup>b</sup>	21	19 ± 12	21	9 ± 5	0.0015
FH	15	23 ± 12	7	13 ± 7	0.003
F-Inv/Depl	6	10 ± 6	14	8 ± 3	NS
Active caspase 3 (sinusoidal) <sup>a</sup>	14	2,013 ± 71	14	2,151 ± 74	< 0.0001
FH	10	1,998 ± 78	4	2,159 ± 55	0.005
F-Inv/Depl	4	2,051 ± 27	10	2,148 ± 83	NS
Apoptosis (TUNEL) <sup>c</sup>	11	12 ± 9	11	9 ± 6	NS

	Before ART		16–20 m of ART		p-value
	n	Baseline values at first LN excision	n	Values at second LN excision	
FH	9	13 ± 10	6	9 ± 8	NS
F-Inv/Depl	2	8 ± 6	5	9 ± 4	NS

LN: lymph node, GC: germinal center, FH: patients with follicular hyperplasia (with or without fragmentation) before therapy and under ART, F-Inv/Depl: patients with follicular involution or depletion before therapy and under ART, NS: not significant, m: months, n: number of patients studied; Peripheral blood: median (range); viral load, lymph node architecture, and markers: mean ± SD (all paired comparisons); <sup>a</sup>Gray level intensity as evaluated by computer assisted image analysis, <sup>b</sup>mean TRAIL<sup>+</sup> cell count/mm<sup>2</sup>, <sup>c</sup>mean apoptotic cell count/GC

follicular hyperplasia (8 without and 12 with fragmentation; representative examples in Figure 1a, c). Ten lymph nodes showed follicular involution and one follicular depletion (Table 2). Primary follicles were detected in only two lymph nodes. The interfollicular T cell area was hyperplastic in most patients (Figure 1e). The medulla was rich in plasma cells, and the sinuses showed a prominent immature sinus histiocytosis. None of the lymph

nodes showed histological signs of opportunistic infections.

After 16–20 months of therapy, the reactive lymph node area and the mean size of the germinal center decreased significantly in all patients (Tables 1, Figure 1b). The number of patients who developed follicular involution or depletion increased after therapy (Table 1 and 2). Of the 20 patients who showed follicular hyperplasia prior to treatment, nine patients still showed this grade after therapy; follicular involution was evident in eight; and follicular depletion in three (Table 2). The lymph nodes of the 11 patients who had follicular involution/depletion initially remained at this histological stage during therapy (Table 2). The number of germinal centers per representative section decreased significantly after therapy only in the group of patients with initial follicular hyperplasia (Table 1). Germinal centers disappeared in the lymph nodes of five patients. Primary follicles reappeared in lymph nodes of 17 patients. The interfollicular T cell area remained cell rich (Figure 1f). The number of CD8<sup>+</sup> T cells that had infiltrated the germinal centers had decreased significantly following therapy in the group that had initially shown follicular hyperplasia (Table 1, Figures 1d, 2h).

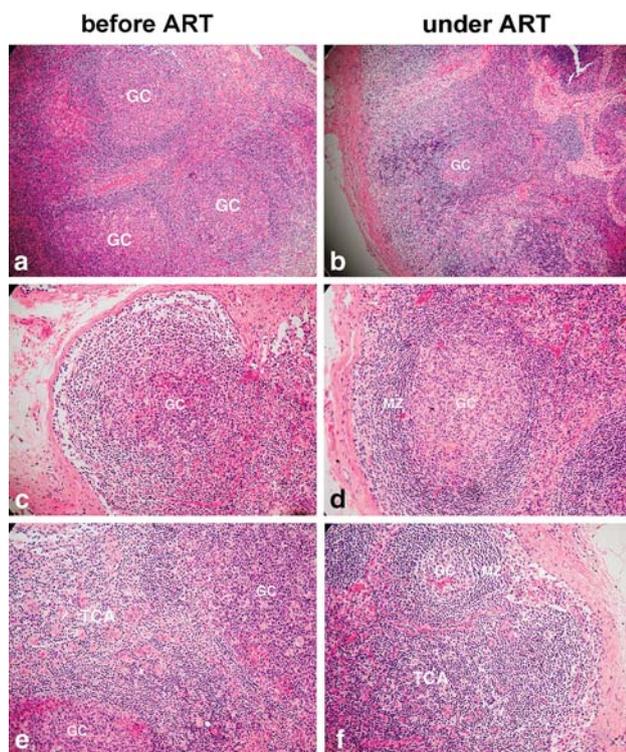
Altogether, after 16–20 months of therapy, the lymph nodes for the majority of patients showed less follicular hyperplasia and more involution/depletion, and the nodes appeared less activated when compared to the corresponding baseline lymph node measurements.

#### Lymph Node Immunostaining

Not all stains on slides could be evaluated because the number of germinal centers either decreased or disappeared following therapy or because of tissue damage.

#### HIV p24-, CD4+ T cell-, CD8+ T cell-, and CD21-Immunostaining

Viral p24 antigen was found only within germinal centers (Figure 2n, o). Prior to treatment, germinal centers in 21 of the 27 investigated patients were p24 positive, and in 6 patients negative. Following therapy, germinal centers in four patients became p24 negative, and the lymph nodes of three patients had lost germinal centers and

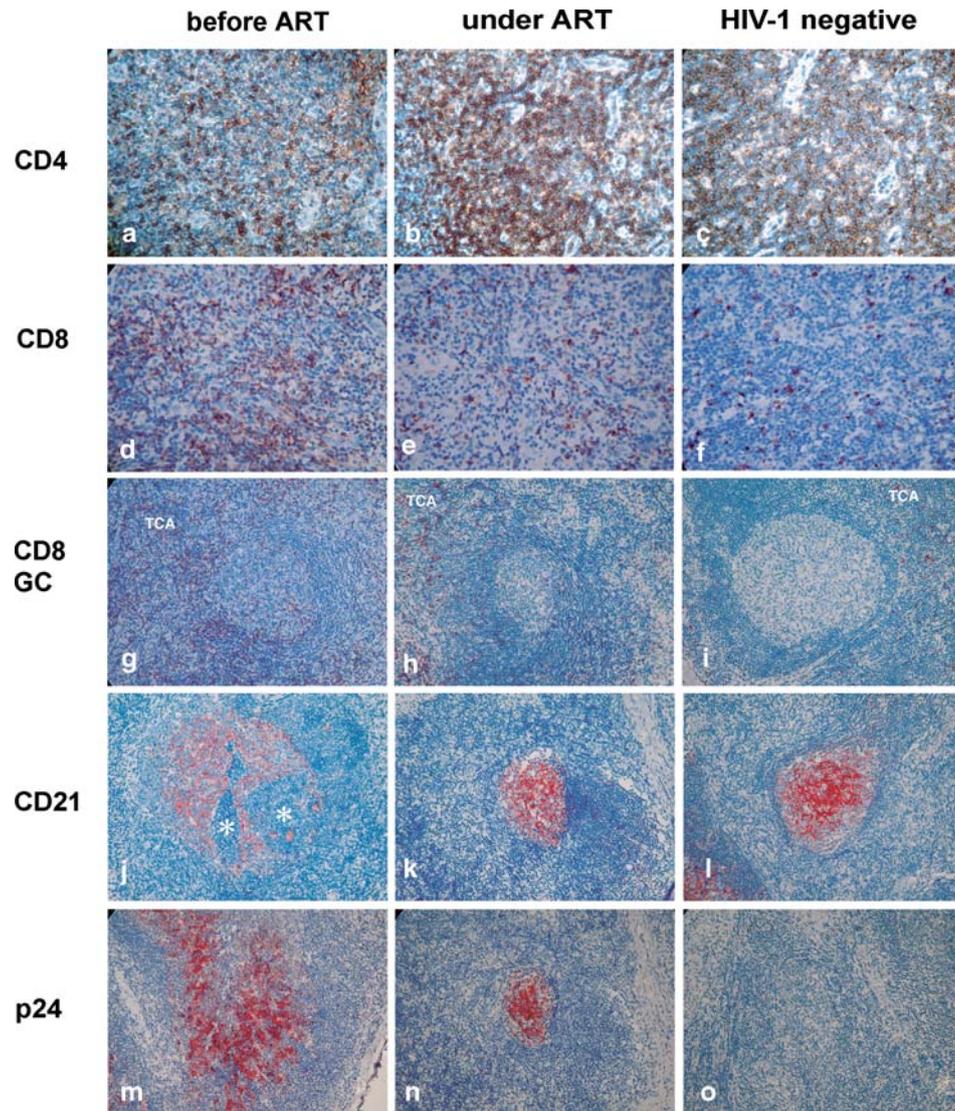


**Figure 1.** Lymph node architecture in an HIV-infected patient before and after antiretroviral therapy (ART). Follicular hyperplasia (FH) decreased after antiretroviral therapy (a, b). Germinal centers (GC) were destroyed by infiltrating cells before therapy and then recovered after antiretroviral therapy (c, d). Note vanishing of mantle zone (MZ) in C and reappearance in D. The T cell area (TCA) was cell rich both before and after antiretroviral therapy (e, f). Hematoxylin–eosin staining, magnifications – a, b: 10×; c–f: 20×.

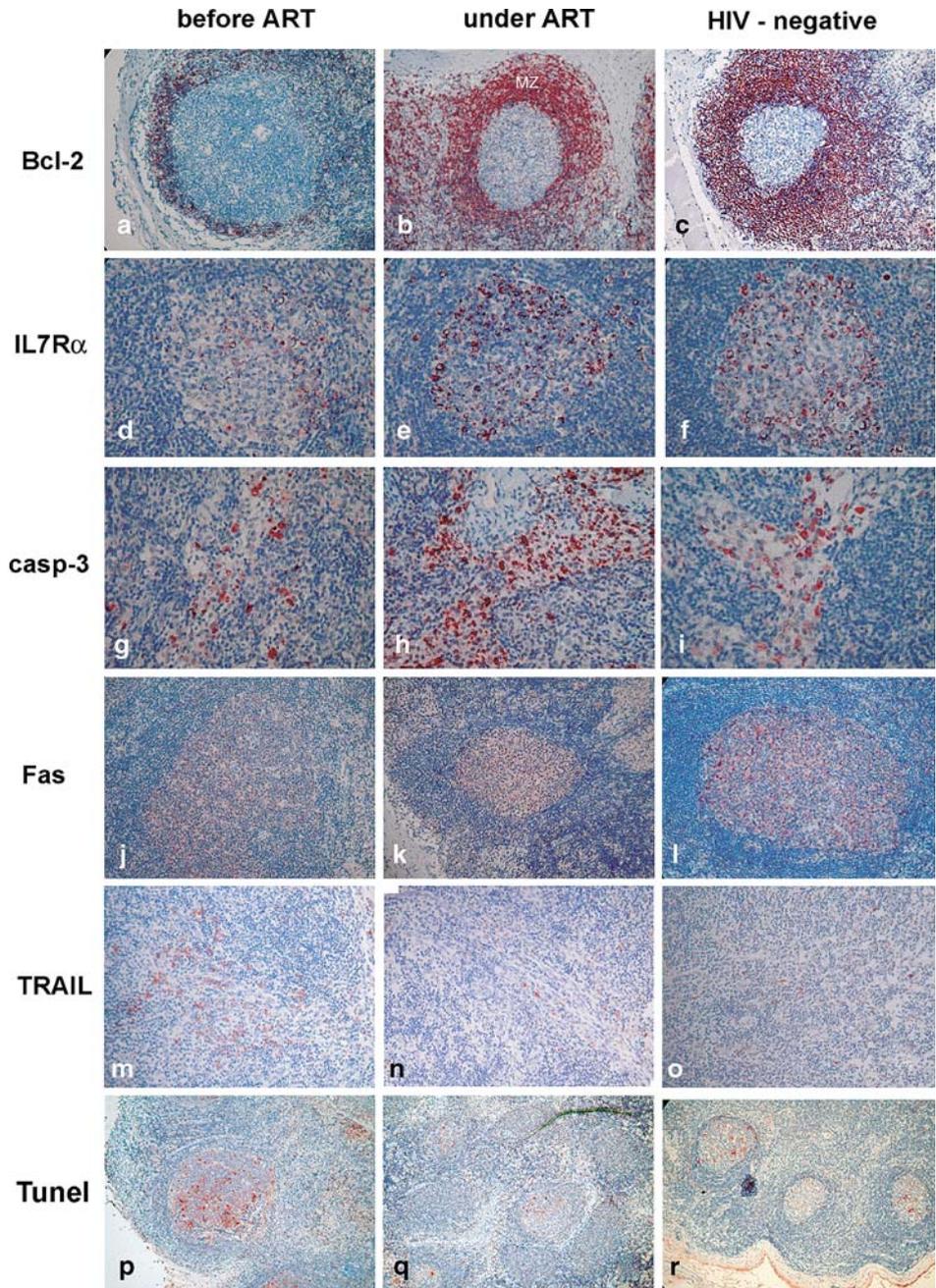
Table 2 Histological grading of lymph nodes before and after antiretroviral therapy.			
Lymph node grading before antiretroviral therapy	n (%)	Lymph node grading after 16–20 months of antiretroviral therapy	n
Follicular hyperplasia	8 (26%)	Follicular hyperplasia	6
Follicular hyperplasia with fragmentation	12 (39%)	Follicular involution	2
Follicular involution	10 (32%)	Follicular hyperplasia	1
Follicular depletion	1 (3%)	Follicular hyperplasia with fragmentation	2
		Follicular involution	6
		Follicular depletion	3
		Follicular involution	9
		Follicular depletion	1
		Follicular depletion	1

n: number of patients

**Figure 2.** Immunohistochemical localization of CD4 (brown DAB reaction product), CD8, CD21, and p24 (red AEC reaction product) in a lymph node of a patient before and after antiretroviral therapy (ART) and in a lymph node of a HIV-negative individual. The CD4+ T cell number interfollicularly increased after antiretroviral therapy (a, b), whereas the number of CD8+ T cells decreased in the T cell area (TCA) (d, e) and within the germinal centers (g, h). The follicular dendritic cell marker CD21 increased after antiretroviral therapy (j, k). Note fragmentation of the red dendritic network by infiltrating lymphocytes (asterisk). p24 persisted in the germinal centers in some of the patients (m, n). The staining in HIV-negative lymph nodes is shown as controls (c, f, i, l, o). Hematoxylin counterstain, magnifications – a–f: 40x; g–p: 20x.



**Figure 3.** Expression of apoptosis and pro-apoptotic and anti-apoptotic markers (AEC red reaction product) in the lymph node of a patient before and after antiretroviral therapy (ART) and in the node of an HIV-negative individual. Bcl-2 positive cells increased in the mantle zone (MZ) after antiretroviral therapy (a, b). IL-7R $\alpha$  expression increased in germinal centers after antiretroviral therapy (d, e). Active caspase 3 increased in the sinuses (g, h), and Fas slightly increased in the germinal centers after antiretroviral therapy (j, k). TRAIL was localized in the sinuses and decreased after antiretroviral therapy (m, n). Apoptosis measured by the TUNEL technique (red stain) was manifested primarily in the germinal centers. In higher magnifications (not shown here), apoptosis was mainly identified as tingible bodies in starry-sky macrophages and as single, scattered apoptotic bodies (p, q). The staining of the same markers is shown in HIV-negative lymph nodes (c, f, i, l, o, r). Hematoxylin counterstain, magnifications: a-c, k-o: 20 $\times$ ; d-i: 40 $\times$ ; p-r: 10 $\times$ .



were p24 negative in the residual lymphoid tissue. In the remaining 14 patients, p24 persisted in the germinal centers after a median time of 18 months post-treatment. The initial six patients with p24-negative germinal centers remained negative. All lymph nodes from HIV-negative controls were negative for p24 staining (Figure 2p).

In the interfollicular area of the lymph nodes, the numbers of CD4<sup>+</sup> T cells increased significantly after therapy (Figure 2a, b), while interfollicular as well as germinal center CD8<sup>+</sup> T cells decreased significantly in all

treated patients (Table 1, Figure 2d, e, g, h), yielding staining comparable to lymph nodes of HIV-negative controls (Figure 2c, f, i).

CD21, a marker for the follicular dendritic network, was weakly distributed over the germinal centers prior to therapy (Figure 2k) and condensed significantly after therapy in 11 of the 12 patients tested (Table 1, Figure 2l), resembling the staining in the germinal centers of HIV-negative controls (Figure 2m). A significant CD21 increase was found only in the patients who had follicular hyperplasia prior to therapy (Table 1).

### Apoptosis in Germinal Centers

Apoptosis as measured in paired lymph nodes obtained from 11 patients was preferentially detected in germinal centers; only a few apoptotic cells were found interfollicularly (Figure 3q, r), very similar as in the lymph nodes of uninfected individuals (Figure 3s). Apoptosis was manifested primarily as tingible bodies in starry sky macrophages as well as in single, scattered apoptotic bodies. Following therapy, a significant decrease in apoptosis was detected in lymph nodes on eight patients ( $p = 0.02$ ), but a slight increase was found in three patients; this yields no statistical significance when all 11 patients were considered together as a group (Table 1).

### Immunostaining of Pro-apoptotic and Anti-apoptotic Molecules

The anti-apoptotic Bcl-2 molecules expressed mainly in B lymphocytes of the mantle zone had significantly increased in all 13 patients who could be evaluated after therapy (Table 1, Figure 3a, b). Only a few Bcl-2 positive cells were found in the germinal centers and in the interfollicular T cell area before and after therapy. IL-7R $\alpha$ , which was expressed mainly in centrocytes and centroblasts of the germinal centers, increased significantly after therapy (Table 1, Figure 3d, e). Active caspase 3 showed a significant increase in the sinuses of the lymph nodes (Table 1, Figure 3k, i) but not in the germinal centers after therapy (not shown). After therapy, staining for these three apoptotic molecules (Figure 3b, e, h) were very similar in intensity and distribution to the staining in HIV-negative control sections (Figure 3c, f, i).

TRAIL staining was detectable in polynucleated granulocytes, in mononuclear histiocytes, and in scattered granular cells that resembled basophils. TRAIL-positive cells were found primarily within vessels and sinuses, some within the interfollicular stroma, and only a few in the germinal centers (Figure 3n, o). The number of TRAIL-positive cells decreased significantly after therapy in the patients who were tested (Table 1). Only a few TRAIL-positive cells were found in the lymph nodes of HIV-negative individuals (Figure 3p).

The increase in Bcl-2, IL-7R $\alpha$ , and active caspase 3 and the decrease in TRAIL were significant only in the group of patients that originally showed follicular hyperplasia in their lymphoid tissue.

The staining of the anti-apoptotic cFlip and the pro-apoptotic Fas (Figure 3k, l) and FasL antigens did not change markedly following therapy and looked similar to staining in the lymph node tissue from HIV-negative individuals (Figure 3m, data not shown).

### Discussion

The patients of this study were in the early stages of HIV infection as indicated by the still high CD4 $^{+}$  T cell counts in the blood ( $> 500$  cells/ $\mu$ l) and the absence of advanced follicular destruction and atrophy in the lymph nodes. We

show that in these patients, antiretroviral therapy, which lowers the viral load and thus reduces the excessively high antigenic stimulation, markedly diminishes the HIV-driven, highly activated immune response in lymph nodes. This is manifested by:

- A marked reduction of follicular hyperplasia: In early HIV infection, follicular hyperplasia is considered to be a physiological reaction of humoral antiviral immunity. The reduction of germinal centers in size and number after therapy fits well with the observed reduction of the viral antigen p24.
- A reconstitution of the follicular dendritic cell network: The disruption and fragmentation of dendritic network and involution of germinal centers are hallmarks of HIV pathology brought about by the abnormal infiltration with CD8 $^{+}$  T cells and their destructive activity on these cells known to carry the virus. The reconstitution of the network corresponds very well with the observed CD8 $^{+}$  T cell reduction and prominent although not always complete loss of antigen on these target cells.
- A reconstitution of Bcl-2-positive mantle zone lymphocytes: The B cell system is affected by HIV infection also at the level of bone marrow derived mantle cells, probably due to the lack of helper factors from CD4 T helper cells. The observed sign of recovery of this committed stem cell compartment indicates a regeneration of the B cell system.
- The appearance of primary follicles: Their presence indicates a revival of primary B immune responses which are obviously exhausted by an exaggerated secondary immune response with germinal center formation before therapy.
- A significant increase in the CD4 $^{+}$  T cell number in the interfollicular area in association with a strong reduction of the CD8 $^{+}$  T cell number in the same area and in the germinal centers. This shift within the two T cell populations in all likelihood reflects the central effect of therapy by reducing viral effects on CD4 T cells and reducing the generation of cytotoxic CD8 cells directed against the former.
- A normalization of the apoptotic markers as evidenced by a distribution of the expression of pro-apoptotic and anti-apoptotic molecules that is similar to that of the uninfected lymph node tissue.

Prior to therapy, the majority of lymph nodes showed reactive follicular hyperplasia, often with giant germinal centers and/or pathologic fragmentation of germinal centers. After 16 to 20 months of therapy, the lymph nodes that were excised from these same patients showed a marked reduction in lymph node and germinal center size and also a significant decrease in the number of germinal centers. Primary germinal centers, which were virtually absent prior to therapy, reappeared in the lymph

nodes of a majority of the patients. In addition, the structure of the germinal centers normalized in most patients – some becoming very small, presumably as a consequence of the reduction of germinal center cells and of CD8+ T cells that had infiltrated the germinal centers and that may have had induced the damage. Because the interfollicular area in these patients remained cell rich, this decrease was likely due to a reduction in immune stimulation rather than to progression to a virus-induced degenerative stage.

HIV persists in the germinal centers throughout the entire course of HIV infection [23], and some viruses remain trapped on the surface of follicular dendritic cell. The antiviral response directly or indirectly (via bystander killing) leads to the destruction of the follicular dendritic cells network as the HIV infection progresses [11]. *Zhang et al.* [11] and *Kuster et al.* [24] reported that with present therapeutic options, complete abolition of viral replication within the lymphoid tissue was not achieved, and *Fischer et al.* [25] showed residual HIV-1 RNA in tonsil biopsy specimen obtained from patients receiving anti-retroviral therapy indicating attenuated viral transcription in HIV infected cells that lack virion production. In our study, from the initial 21 patients with p24 positive lymph nodes, only 7 became p24 negative during therapy, while the plasma viral load decreased significantly in all 31 patients.

In agreement with results of other studies [11, 26, 27], the follicular dendritic cell network recovered after therapy, as shown by a significant increase in CD21. In most of our patients, however, the follicular dendritic cell network was not completely disrupted prior to therapy, presumably because the patients were still in an early stage of HIV infection.

CD8+ T cells are the key effectors of the antiviral defense, thereby mediating the destruction taking place in the lymph nodes. *Blackburn et al.* [28] reported that the extent of anti-HIV activity by CD8+ T cells in the lymphoid tissue correlated with the viral burden. We found a high number of CD8+ T cells in the interfollicular area and within the germinal centers before treatment, followed by a significant decrease after treatment. Consistent with other studies [27, 29, 30], the CD4+ T cell numbers in the lymph nodes increased significantly after therapy.

The lowering of the highly activated immune response is also reflected by the normalization of some important pro- and anti-apoptotic markers. The expression of Bcl-2, IL-7R $\alpha$ , TRAIL, and active caspase 3 was different in the lymph nodes that were excised 16–20 months after treatment and markedly resembled expression in non-infected individuals. Moreover, in 8 of the 11 patients who were tested for these markers, apoptosis decreased after therapy, which indicated that apoptotic processes may have normalized with therapy. The decrease in apoptosis in germinal centers combined with the fact that active caspase 3 was only increased in

the sinuses but not in germinal centers indicates a caspase-independent cell death pathway in germinal centers. Indeed, it has been shown that productive HIV infection can lead to caspase independent cell death [31].

Only a few other investigators have looked for apoptotic markers and apoptosis in lymph nodes; most have looked for them in peripheral blood lymphocytes. Interestingly, cellular distribution of several molecules seems to differ in lymph node lymphocytes and peripheral blood lymphocytes, although this may be in part due to technical reasons. The peripheral blood markers were determined by FACS analysis, while the markers in the lymph nodes were identified by immunohistochemistry. For example, although Bcl-2 was found to be expressed in peripheral T-lymphocytes and strongly correlated with spontaneous T cell apoptosis [32–35], in lymph nodes, Bcl-2 was detected primarily in the B (mantle zone of the germinal centers) and not in the T cell area. Similarly, IL-7R $\alpha$  was found to be expressed on peripheral T and B cells, and peripheral CD4+ T cell depletion correlated well with the down-regulation of IL-7R $\alpha$  [36, 37]. In lymph nodes, IL-7R $\alpha$  which increased after therapy was detectable only in the germinal centers and not in the T cell area. The absence of IL-7R $\alpha$  expression in the T cell area resembles those peripheral T cells in which a strong association of decreased IL-7R $\alpha$  expression with low Bcl-2 expression has been found [36, 37]. In lymph nodes, a significant down-regulation of TRAIL was found after therapy due primarily to the reduction of cells of the phagocytic type, whereas TRAIL was not identifiable in B or T lymphocytes. In peripheral blood, TRAIL has been discussed as a major death-ligand in HIV infection [38]. Thus, TRAIL, but not FasL-dependent AICD, was found in peripheral CD4+ T cells that were isolated from HIV-infected individuals [39, 40]. Whether the CD4+ T cell recovery in lymph nodes after therapy is the consequence of the marked decrease of TRAIL-positive cells is unknown. When the apoptotic and histological markers were correlated, significant differences before and after therapy were found only in the group of patients with follicular hyperplasia. The failure to demonstrate significant differences before and after therapy in the follicular involution/depletion group may be due to the less numbers of patients tested in this study.

This study has some limitations: (a) Untreated patients who were in the early stages of HIV infection were selected for this study, but we have no comparison group of untreated patients in an advanced stage of HIV infection. (b) For ethical reasons, there is no matched control group of untreated HIV patients in early stages of HIV infection. (c) For technical reasons, apoptosis could only be followed sequentially in the lymph nodes of one-third of the patients in the study, and the apoptotic markers that were measured reflect indirect apoptotic processes.

However, because histological assessments were done in parallel, we believe that the results of the study are valid. The strength of the study is the considerable number of patients from whom sequential lymph node tissue samples have been investigated, together with the analysis of a comprehensive number of immunological, virological, and histological parameters.

In conclusion, two processes might be involved in the current therapeutic setting of these patients: (a) a progression of degenerative changes in the germinal centers as part of the virus-induced pathology in spite of therapy, and (b) the physiological reduction of a hyperimmune response as a result of therapy. Conventional histology indicates that viral damage prevails after involution has been established. The arrest of viral pathology and normalization of follicular hyperplasia appears to prevail in the stage of follicular hyperplasia. However, even in cases of persistent involution, there are signs of restoration, such as repopulation of the mantle zone, restoration of the dendritic net, normalization of apoptotic markers, and the appearance of primary follicles.

Our data are consistent with observations made in patients with primary HIV infection who were treated with antiretroviral therapy and cyclosporin A [41]. Rapid shutdown of T cell activation by cyclosporin A in the early phase of HIV infection proved to be beneficial for both immunological and virological measures by preventing ongoing infection of new target cells in the lymphoid tissue [2, 42]. Recent reports have shown that immune reconstitution in the intestinal lymphoid tissues did not take place after antiretroviral therapy in acute and early stage HIV infection [43, 44]. Because the intestinal lymphoid system, as opposed to the inguinal lymph nodes, is constantly exposed to a huge number of different antigens, the continuously overactivated immune response may be more prone to the damaging effect of HIV infection. Therefore, HIV pathogenesis may be different in the intestinal lymphoid tissue and inguinal lymph node tissue.

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