Restoration of *Toxoplasma gondii*-specific immune responses in patients with AIDS starting HAART

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Objective: To study the kinetics and identify factors associated with *Toxoplasma*-specific immune responses in patients with AIDS starting antiretroviral therapy.

Methods: A prospective study of 38 HIV-infected patients seropositive for *Toxoplasma* who started antiretroviral therapy with CD4 T cells less than 200 cells/μl. T-cell and B-cell phenotypes, anti-*Toxoplasma* antibodies titers, Th-1 and Th-2 cytokine production and lymphocyte proliferative responses (LPRs) to *Toxoplasma* were assessed over 12 months.

Results: Median CD4 cell count increased from 122 cells/μl at baseline to 260 cells/μl at 12 months, and the incidence of a positive LPR from 18.4 to 70.5%. A *Toxoplasma* IgG titer more than 150 IU/ml was the only baseline variable associated with a positive LPR (hazard ratio: 4.6, $P = 0.003$). Among time-dependent covariates, the number of effector memory (CD45RA−CCR7−) CD4 T cells was associated with a positive LPR ($P < 0.02$) and the number of terminally differentiated (CD45RA+CCR7−) CD8 T cells was associated with in-vitro production of γ-IFN ($P < 0.008$).

Conclusion: Among patients with low CD4 cell counts, high anti-*Toxoplasma* IgG titers were associated with LPR to *Toxoplasma* antigen. After starting antiretroviral therapy, the number of effector memory CD4 T cells and terminally differentiated CD8 T cells were associated with the restoration of *Toxoplasma* LPR and γ-IFN production, respectively.

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Introduction

The introduction of HAART has led to a rapid decline in morbidity and mortality among patients with advanced HIV infection [1]. This benefit was related to a dramatic decrease in the incidence of most opportunistic infections following immune reconstitution in patients with low CD4 T-cell counts [2]. Indeed, suppression of viral replication under HAART induces a rapid rise in CD4 T cells and a restoration of antigen-specific immune responses to several antigens such as cytomegalovirus, *Candida albicans*, *Mycobacterium tuberculosis* and *Mycobacterium avium* [3–6]. A similar restoration of immune responses to *Toxoplasma gondii*, a major opportunistic pathogen in patients with AIDS, has also been reported in patients receiving HAART [7]. A number of cohort studies and clinical trials [8–11] have shown that in patients with high CD4 T cells and full inhibition of HIV replication under HAART, primary and secondary *T. gondii* prophylaxis could be safely discontinued. Current

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recommendations suggest indeed that a CD4 T-cell count above 200 cells/μl for at least 3 months will allow the discontinuation of such prophylaxis [12,13]. However, most studies addressing the issue of *T. gondii* prophylaxis discontinuation in patients with HIV infection have enrolled patients with CD4 T-cell counts above 200 cells/μl for more than 8–12 months, with a median duration of HAART of 1–2 years and with a median CD4 T-cell counts at the enrolment usually above 300 cells/μl. Furthermore, no analysis of *T. gondii*-specific immune responses was performed in these studies and few data are available on the time course of *T. gondii*-specific immune reconstitution in patients with advanced HIV infection [7,14–16].

In this study, we wished to prospectively assess the kinetics of *T. gondii*-specific immune responses in patients with AIDS starting HAART, using in-vitro lymphocyte proliferation and cytokine production assays in presence of *T. gondii* antigen, and to identify predictive factors associated with this immune reconstitution.

Patients and methods

The present study was conducted in the Department of Infectious Disease of the Saint-Louis Hospital, Paris, France. It was a prospective single-centre cohort study, conducted from December 2002 to June 2005. HIV-1-infected patients with a positive *Toxoplasma* serology, naive to antiretroviral therapy and with a baseline CD4 cell count of less than 200 cells/μl were enrolled consecutively in this study. Patients with uncontrolled opportunistic infections, receiving treatment with steroids or chemotherapy, were excluded. All patients started HAART at baseline. Demographic characteristics (age, sex, HIV risk factor, and Centre for Disease Control stage) of the patients were recorded at enrolment and blood samples were taken for plasma HIV RNA levels, *Toxoplasma* serology, flow cytometry analysis of T-cell and B-cell, lymphocyte proliferation assays and cytokine production every 3 months for 12 months. Thirty patients were recruited in this study. Eighteen non-HIV-infected healthy volunteers with a positive anti-*Toxoplasma* serology served as normal controls. All persons gave written informed consent to participate to this study, which was approved by the Paris Saint-Louis ethics committee.

Plasma viral RNA-assay

Plasma HIV-1 RNA load was measured by an ultrasensitive PCR assay (Ultrasensitive Amplicor HIV-1 1.5 Monitor; Roche Molecular Systems, Branchburg, New Jersey, USA) with a lower limit of quantification of 50 copies/ml.

Toxoplasma serology

IgG and IgM antibodies were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit (BioRad, Marnes La Coquette, France) and by direct sensitized agglutination (Toxoscreen; Bio-Mérieux, Marcy L'Etoile, France). IgG titers were expressed in IU/ml. A serology was considered positive for IgG titers 6 IU/ml at least with a positive agglutination test.

Western blots were also performed at each visit to analyse the specificity of antibodies and to detect the emergence of new bands using a commercial kit (LDBio Diagnostics, Lyon, France).

Flow cytometry analysis

Lymphocyte immunophenotyping was performed on fresh whole blood EDTA samples by direct three-colour or four-colour immunofluorescence, using appropriate monoclonal antibodies (Becton Dickinson, San Jose, California, USA, and Coulter-Beckman-Immunotech, Villepinte, France). Ten thousand gated lymphocytes were analysed with a Facscalibur analyser (Becton Dickinson). Lymphocyte gate purity was 98% at least. Characterization of central memory (CD45RA−CCR7+), effector memory (CD45RA−CCR7−), terminally differentiated (CD45RA+CCR7−), competent (CD28+) and naive (CD45RA+CCR7+) T CD4 and CD8 cells subsets, and naive (CD19+CD27−) and memory (CD19+CD27+) B cells subsets were assessed using standardized procedures.

Lymphocyte proliferation assay

Cultures of peripheral blood mononuclear cells isolated from sodium heparinate blood samples by gradient density were performed in 96-well microtiter plates, in triplicate, at 10^5 cells per well with 0.5 μg/ml of soluble antigen extract of *T. gondii* (SAT), prepared from trophozoites of the RH strain as previously described, or medium alone [7]. Phytohemagglutinin (PHA) (Murex Biotech, Dartford, UK) 0.5 μg/ml + IL-2 (10 IU/ml) (Boehringer, Mannheim, Germany) was used as a control. Lymphocyte proliferative responses (LPRs) were quantified by incorporation of tritium-labelled thymidine (1 μCi/ml; Amersham, Buckinghamshire, UK) for 18 h in 7 days triplicate cultures. Results were expressed as mean counts per minute (cpm) per 10^5 cells. The stimulation index was calculated for each sample as the ratio of cpm in stimulated versus unstimulated cultures. A stimulation index equal or greater than 5 associated with a cpm count 5000 at least defined a positive antigen-specific response.

Cytokine production and dosage

Th-1 (IL-2, TNF-α, γ-IFN) and Th-2 (IL-4, IL-10, IL-5) cytokine production was quantified in mononuclear cell supernatants stimulated for 48 h with SAT (*Toxoplasma* antigen) or medium alone (unstimulated wells). Stimulation with anti-CD3+ and anti-CD28+ antibodies served as a positive control. Supernatants were then harvested and stored at −80°C until analysed. Cytokine production was measured with a CBA kit, according to the manufacturer's procedure (Becton

Dickinson). The concentrations of the six cytokines were determined simultaneously in the same supernatants. Results were expressed as Δ pg/ml of cytokine (Δ = mean production in stimulated − mean production in unstimulated wells). Six standard curves, one for each cytokine, were generated ranging from 0 to 5000 pg/ml. According to these curves, concentrations of 10 pg/ml or more were considered significant for the production of γ-INF, and concentrations of 5 pg/ml or more were considered significant for the production of the other cytokines.

Statistical analysis

Results were reported as median [25th–75th percentiles] for continuous variables and counts (percentages) for categorical variables. Lymphocyte subsets and cytokine productions were presented as box plots where boxes extend from the 25th to 75th percentiles, lines inside the boxes represent the median value, and whiskers represent fifth and 95th percentiles. Tests for trends were performed to assess significant changes over time using the Cochran–Armitage test for trend. Comparisons across groups used χ^2 test or Fisher's exact test when appropriate for categorical variables, and Kruskall–Wallis test for continuous variables. A multivariable logistic regression analysis was performed to assess the influence of the baseline covariates on the LPR to *Toxoplasma* antigen. Influences of time-dependent covariates on LPR to *Toxoplasma* antigen and γ-IFN production were assessed through the use of mixed model. The relationship between *Toxoplasma* antigen proliferation and Th-1 cytokine production used Spearman's correlation. All tests were two-tailed. All computations were done using SAS software (SAS Institute Inc., Cary, North Carolina, USA).

Results

Patients' characteristics and follow-up

Thirty-eight HIV-infected patients were enrolled in the study. Baseline characteristics of these 38 HIV-infected patients are shown in Table 1. Median baseline CD4 cell count was 122 cells/μl, and median plasma HIV RNA level was 4.82 $\log_{10}$ copies/ml. Thirty-three of 38 patients (87%) started a protease inhibitor-containing regimen, mostly lopinavir/ritonavir ($n = 16$) or nelfinavir ($n = 14$). The most frequently used nucleoside reverse transcriptase inhibitor combination was zidovudine + lamivudine (25 of 38 patients, 66%). One patient never started HAART. During the 12-month period of the study, eight patients were lost to follow-up or withdrew consent and one died of a myocardial infarction. No case of cerebral toxoplasmosis was reported but two patients experienced an AIDS-defining event (Kaposi's sarcoma and non-Hodgkin's lymphoma). At month 12, 23 of 29 patients (79%) patients with available data had plasma HIV RNA levels below 400 copies/ml and median CD4 cell count was 260 cells/μl.

Healthy controls were younger, more frequently female, and with a higher CD4 cell count than patients at baseline but had a similar level of anti-*Toxoplasma* antibodies (Table 1).

B-cell and T-cell phenotypes in peripheral blood

CD4 and CD8 T-lymphocytes subsets over time are depicted in Fig. 1a and b.

There was a significant increase in all CD4 subsets over time, from 23, 49, 48, 3 and 103 cells/μl at baseline to 64, 102, 71, 7 and 225 cells/μl at month 12 among naive, central memory, effector memory, terminally differentiated and competent subsets, respectively. Corresponding numbers in controls were 400, 225, 179, 12 and 100 for naive, central memory, effector memory, terminally differentiated and competent subsets, respectively. Among CD8 subsets, only competent, naive, terminally differentiated and central memory subsets significantly increased over time. There was also an increase in naive and total B cells over time (Fig. 1c).

Lymphocyte proliferative responses to soluble antigen extract of *Toxoplasma gondii*

All the 38 HIV-infected patients had a positive proliferative response to PHA. The cumulative incidence of positive LPR to SAT in the 38 patients over time is

Table 1. Characteristics of HIV-infected patients and non-HIV-infected healthy controls.

Median [25th–75th percentiles] number (%)	Patients $n = 38$	Controls $n = 18$	*P*
Age	43 [37–50]	35 [25–42]	0.0050
Sex male	33 (87%)	3 (17%)	<0.0001
Risk group			
Heterosexual	17 (45%)	NA	
Homosexual	17 (45%)	NA	
Drug user	3 (8%)	NA	
CDC[a] clinical stage C	15 (39.5%)	NA	
Toxoplasma prophylaxis	32 (84%)	0	
Plasma HIV RNA level ($\log_{10}$ copies/ml)	4.82 log [4.38–5.55]	NA	
CD4 cell count (cells/μl)	122 [51–194]	863 [664–1205]	<0.0001
Anti-*Toxoplasma* IgG (IU/ml)	192 [56–1410]	128 [55–224]	0.14

P values are reported for comparison between the groups, based on either exact Fisher test or Wilcoxon sum rank test. NA, not applicable.
[a]Centre for Disease Control.

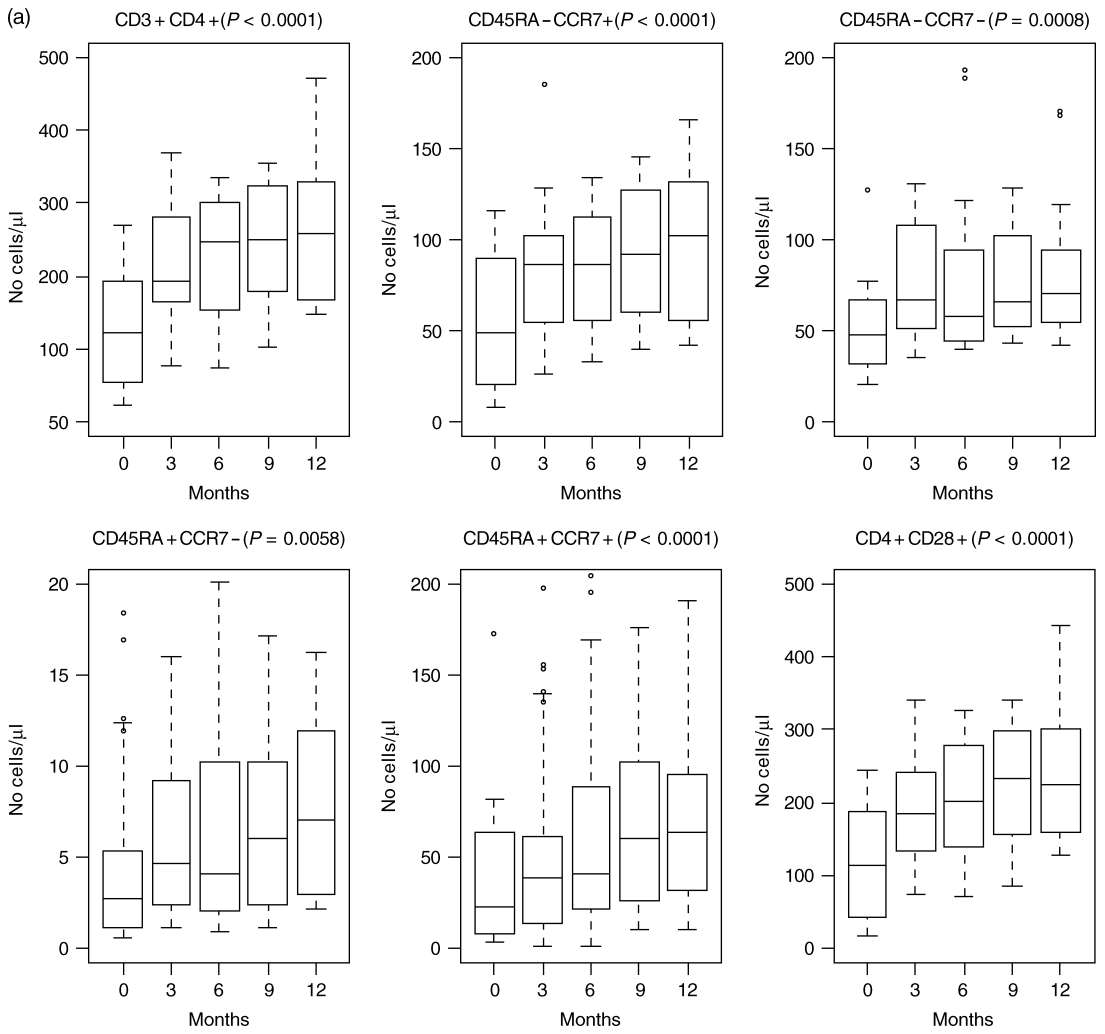


Fig. 1. Box plots of CD4 and CD8 T-lymphocytes subsets over time. (a) Box plots of CD4 T cells subsets (cells/μl) over time in HIV-infected patients starting HAART (*P* value for time trend): central memory (CD45RA−CCR7+), effector memory (CD45RA−CCR7−), terminally differentiated (CD45RA+CCR7−), competent (CD28+) and naive (CD45RA+CCR7+) subsets. (b) Box plots of CD8 T-cells subsets (cells/μl) over time in HIV-infected patients starting HAART (*P* value for time trend): central memory (CD45RA−CCR7+), effector memory (CD45RA−CCR7−), terminally differentiated (CD45RA+CCR7−), competent (CD28+) and naive (CD45RA+CCR7+) subsets. (c) Box plots of B cells subsets (cells/μl) over time (months) in HIV-infected patients starting HAART (*P* value for time trend): naive (CD19+CD27−) and memory (CD19+CD27+) subsets.

shown in Fig. 2; it increased from 18.4% at baseline to 70.5% at month 12. Of note, at month 12, the prevalence of positive LPR to SAT in cases (19 out of 29, 65.5%) remained lower than in controls (100%, $P = 0.008$). Stimulation indexes among responders were similar in patients and controls (data not shown).

Cytokines production after soluble antigen extract of *Toxoplasma gondii* stimulation

Production of Th-1 cytokines (IL-2, γ-IFN and TNF-α) and Th-2 cytokines (IL-4, IL-5 and IL-10) are shown in Table 2. Overall, very few patients and controls produced Th-2 cytokines after SAT stimulation. In contrast, the production of Th-1 cytokines after SAT stimulation was readily detectable in tissue culture supernatants, but significantly fewer patients than controls produced these cytokines, with no significant trend over time in patients (Table 2). Among Th-1 cytokines detected in the supernatants following antigenic stimulation, IFN-γ and TNF-α were the main cytokines detected.

Of note, patients had a spontaneous production of TNF-α at baseline (median of 54 pg/ml) with a significant decrease over time to normal values (data not shown).

Also, the production of γ-IFN, TNF-α and IL-2 in presence of SAT was significantly correlated with the LPR stimulation index to SAT, with Spearman's

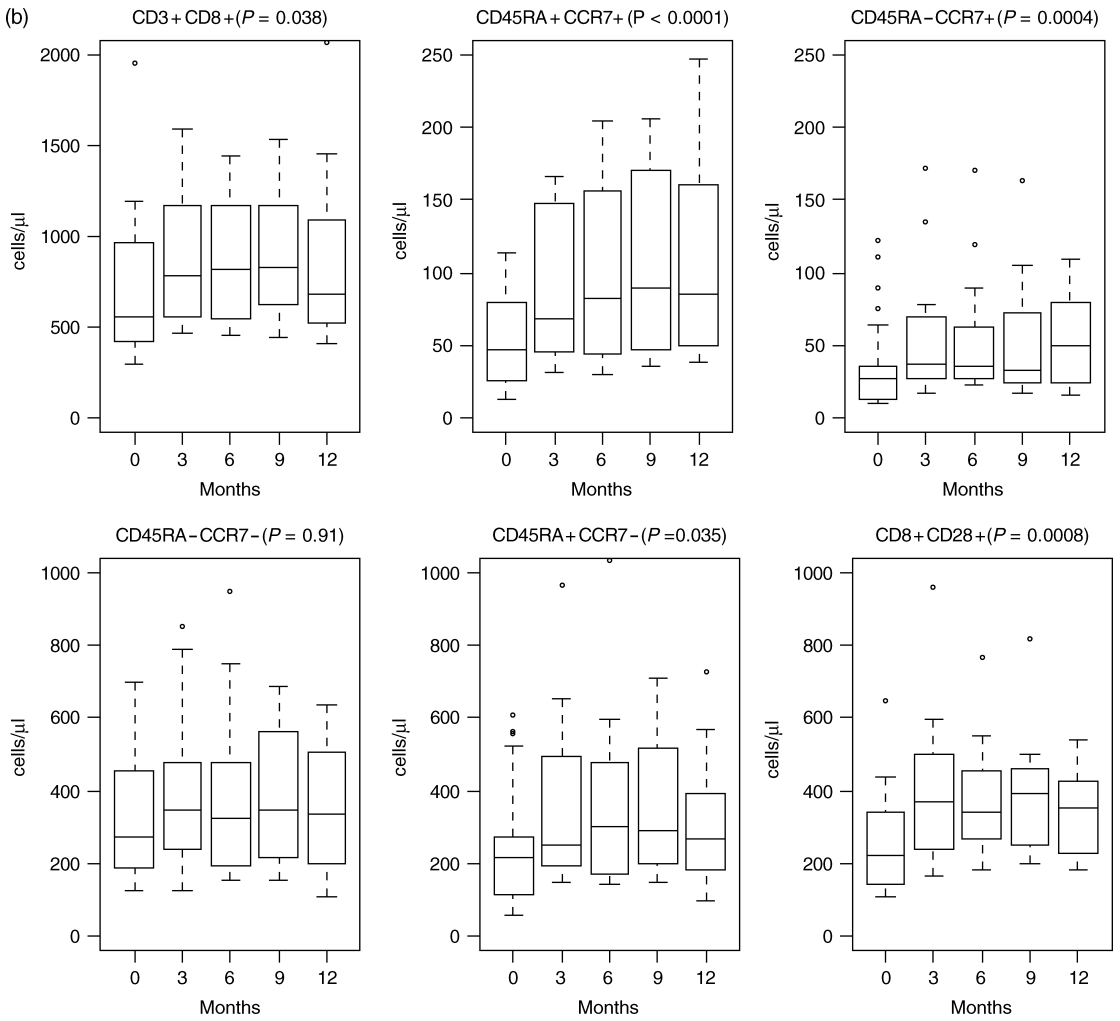


Fig. 1. (*Continued*).

correlation coefficient estimated at 0.53 ($P < 10^{-4}$), 0.21 ($P = 0.007$) and 0.61 ($P < 10^{-4}$), respectively.

Production of anti-*Toxoplasma* antibodies

As compared with baseline values, no significant change in antibody production was noted over time among HIV-infected patients with no emergence of new band on western blots (Fig. 3a and b).

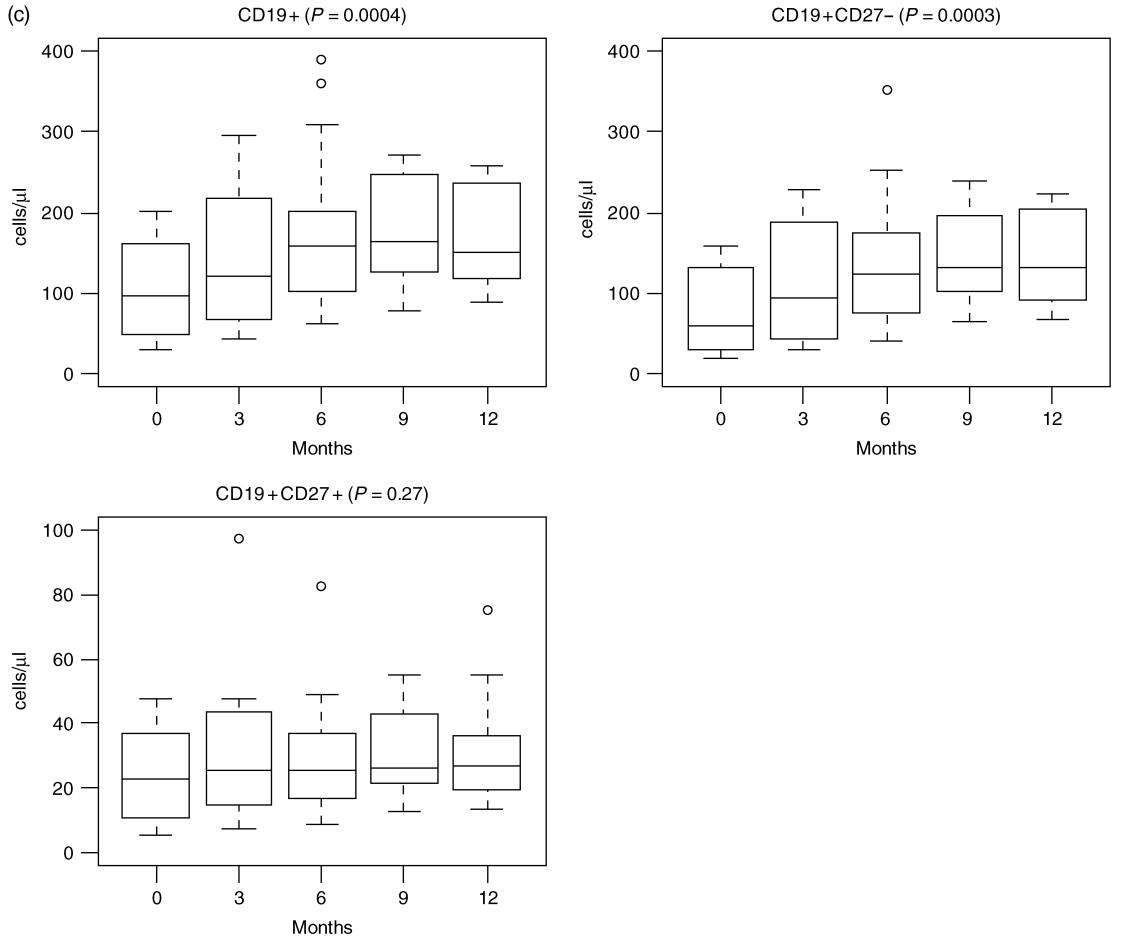
Predictive factors associated with *Toxoplasma*-specific lymphocyte proliferation and γ-interferon production

Baseline-dependent and time-dependent prognostic factors associated with a positive LPR response are shown in Tables 3 and 4, respectively. At baseline, only an anti-*Toxoplasma* antibody titer above 150 IU/ml was significantly associated with a positive LPR with a hazard ratio = 4.61, 95% confidence interval (CI) [1.66–12.8], $P = 0.003$. Among time-dependent variables, only the number of effector memory CD4 T cells (CD45RA−CCR7−) was associated with a positive LPR [OR = 1.02 per cell increase, 95% CI (1.004–1.036), $P = 0.02$].

Similar analyses were performed to identify variables associated with the production of γ-IFN over time (Table 4). Based on univariate analyses, response of γ-IFN was correlated with lymphocytes count, the number of effector memory CD4 T-cells, of CD8 cells and of terminally differentiated CD8 cells. When introduced in a multivariate model, only the number of terminally differentiated CD8 cells remained significant [OR = 1.0022 per cell increase, 95% CI (1.0006–1.0030), $P = 0.0008$].

Discussion

The depletion of T lymphocytes is required for reactivation of *T. gondii* infection in humans, and a low



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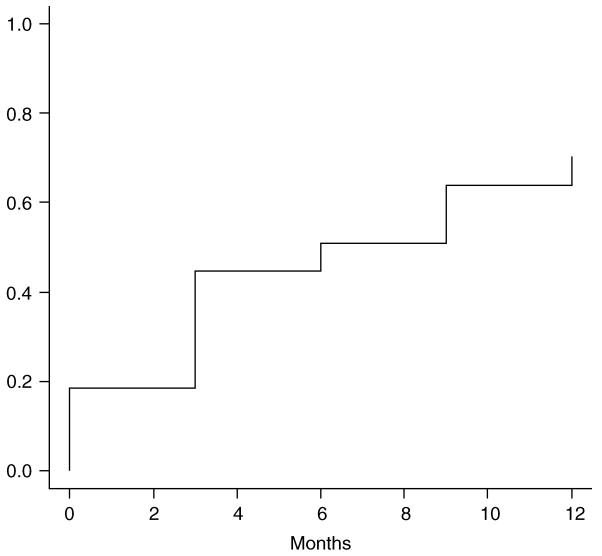

Fig. 2. Estimated cumulative incidence of positive lymphoproliferative responses to *Toxoplasma gondii* antigens over time in HIV-infected patients starting HAART.

CD4 T-cell count has been identified as a major risk factor for Toxoplasma encephalitis in patients with HIV-infection [17–19]. Indeed, the production of γ-IFN, a cytokine produced by CD4 and CD8 T cells, plays a major role in the defence against *T. gondii* infection and is decreased in patients with advanced HIV-infection [20–23].

The results of our study, therefore, confirm that among patients with low CD4 cell counts (less than 200 CD4 T cells per µl), there is a profound defect in γ-IFN production and lymphocyte proliferation in presence of *T. gondii* antigens [7,14–16]. These patients should, therefore, receive a primary prophylaxis in order to prevent *T. gondii* reactivation. However, a small proportion of these patients in our study could demonstrate at baseline in-vitro *T. gondii*-specific immune responses with significant lymphocyte proliferation and γ-IFN production following antigenic stimulation (Table 2, Fig. 2). When analysing the different baseline factors associated with lymphocyte proliferation, only a high anti-*Toxoplasma* antibody titre above 150 IU/ml was

Table 2. Proportion of HIV-infected patients and controls with Th-1 (γ-IFN, IL-2, TNF-α) and Th-2 (Il-4, IL-5, IL-10) cytokine production following *Toxoplasma gondii* antigenic stimulation.

Cytokines	Baseline ($n=33$)	M3 ($n=32$)	M6 ($n=31$)	M9 ($n=30$)	M12 ($n=29$)	Controls ($n=17$)	*P* value time trend in cases	*P* value cases M12 versus controls
γ-IFN ≥10 pg/ml	5 (15.1)	11 (34.4)	16 (51.6)	11 (36.7)	8 (27.6)	11 (64.7)	0.26	0.014
IL-2 ≥5 pg/ml	1 (3.0)	10 (31.2)	9 (29.0)	4 (13.3)	6 (20.7)	12 (70.6)	0.39	0.0008
TNF-α ≥5 pg/ml	12 (36.4)	10 (31.2)	9 (29.0)	10 (33.3)	9 (31.0)	13 (76.5)	0.73	0.003
IL-4 ≥5 pg/ml	0	1 (3.1)	2 (6.4)	1 (3.3)	0	4 (23.5)	0.93	0.015
IL-5 ≥5 pg/ml	0	0	1 (3.2)	1 (3.3)	0	1 (5.9)	0.57	0.37
IL-10 ≥5 pg/ml	5 (15.1)	0	1 (3.2)	3 (10.0)	0	2 (11.8)	0.12	0.13

Cytokine responses are expressed as Δ pg/ml: mean (pg/ml) in wells stimulated with *Toxoplasma* antigen − mean (pg/ml) in the unstimulated wells. Values are the number (%) of responders over time. Data in parentheses are in percentage.

identified with an odds ratio of 4.61 ($P=0.003$). This result, therefore, confirms previous observations [7], though high anti-*Toxoplasma* IgG titres were also found to be predictive of the occurrence of toxoplasmic encephalitis in patients with a CD4 cell count below 200 μl^{-1} [18,24]. We have no clear explanation for these discrepancies, except that previous studies were performed before the HAART era in patients with CD4 cell counts progressively declining. Subclinical reactivation of dormant cysts may have occurred in our chronically infected patients and may have been responsible for a secondary type humoral response to the recall antigen [24]. Elevated levels of anti-*Toxoplasma* IgG titre could, therefore, reflect a good immune response to *Toxoplasma* antigen, both humoral and cellular. However, the clinical consequences of these detected *T. gondii* immune responses in patients with low CD4 cell counts remained to be determined.

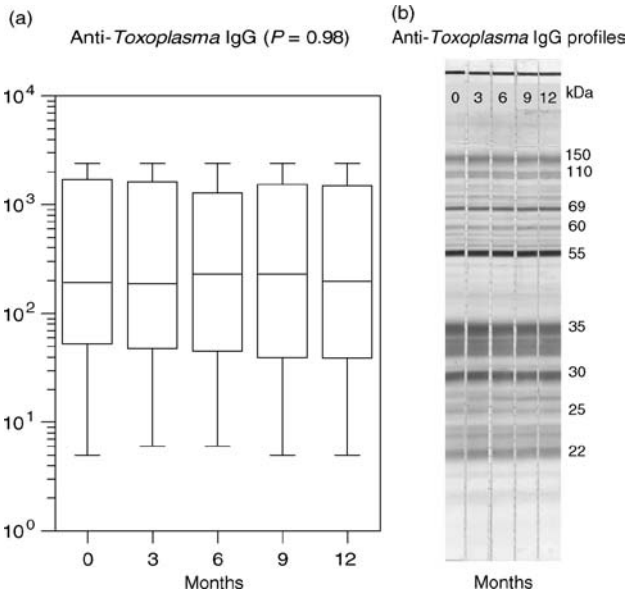

Fig. 3. Anti-*Toxoplasma* IgG antibodies in HIV-infected patients starting HAART. (a) Boxplot of IgG titers (UI/ml) over time (months). (b) Representative IgG western blot profile of a patient over time (months).

Following the initiation of HAART in our study, we observed as expected significant increases in all CD4 T-cell subsets and in most of the CD8 T-cell subsets (Fig. 1a and b). Only naive and not memory B-cell subsets increased on HAART, however, which might explain the unchanged anti-*Toxoplasma* IgG titre over time in this study, with no visible change in the specificity of the antibodies (Figs 1c and 3).

After only 12 months of HAART, T-cell responses to *T. gondii* antigens were restored in most patients with 65.5% having positive lymphoproliferative responses, whereas median CD4 T-cell counts was only 260 cells/μl and 79% had controlled viral replication (Fig. 2). These patients could be eligible for an early discontinuation of anti-*Toxoplasma* prophylaxis. However, a significant proportion of our patients still lacked sufficient immune responses after 12 months of HAART and remained at risk for *T. gondii* reactivation. Also, the proportion of patients with in-vitro production of γ-IFN after 12 months of HAART remained twice lower than controls. This was also seen with other Th-1 cytokines (IL-2 and TNF-α), whereas Th-2 cytokines were rarely produced following *T. gondii* exposure in both patients and controls (Table 3). A careful monitoring of such patients should, therefore, be recommended before discontinuing prophylaxis, whatever their CD4 cell counts could be. Indeed, a number of reports have shown that among patients with a low CD4 cell count nadir, immune defects to specific pathogens could persist despite restoration of high CD4 cell counts, putting them at risk for reactivation of opportunistic infections [25–30]. Therefore, increased CD4 T-cell counts under HAART are not always associated with the restoration of immune responses to opportunistic pathogens, and it would be interesting to have better predictors of immune reconstitution. Interestingly, we have found in our study that the restoration of effector memory CD4 T cells was associated with *T. gondii*-specific lymphocyte proliferation (Table 4). This finding is not unexpected, however, as effector memory dysfunction has been previously associated with susceptibility to opportunistic infections among HIV-infected patients [3,5,16,28,29]. Also, effector memory CD4 T cells are among CD4 T-cell

Table 3. Baseline predictive factors of time to first positive lymphoproliferative response to *Toxoplasma* antigen among HIV-infected patients.

	Hazard ratio of response (95% CI)	*P*
Age	1.011 (0.975–1.048)	0.55
Sex male	0.70 (0.24–2.07)	0.52
Plasma HIV RNA level (copies/ml)	1 (1–1)	0.88
Toxoplasma IgG titer >150 IU/ml	4.61 (1.66–12.8)	**0.003**
Lymphocytes count (cells/μl)	1.000 (1.000–1.001)	0.41
CD4 cells (cells/μl)	1.003 (0.999–1.007)	0.13
Central Memory CD4 cells	1.002 (0.993–1.01)	0.66
Effector memory CD4 cells	1.01 (0.995–1.024)	0.19
Terminally differentiated	1.036 (0.979–1.096)	0.22
Naive CD4 cells	1.004 (0.995–1.013)	0.39
Competent CD4 cells	1.003 (0.999–1.007)	0.16
CD8 cells (cells/μl)	1 (0.999–1.001)	0.63
Central memory CD8 cells	1.003 (0.988–1.017)	0.72
Effector memory CD8 cells	1 (0.999–1.002)	0.73
Terminally differentiated CD8 cells	1 (0.999–1.002)	0.65
Naive CD8 cells	1.002 (0.993–1.012)	0.66
Competent CD8 cells	1.001 (0.999–1.004)	0.39
B cells (cells/μl)	0.999 (0.994–1.005)	0.76
CDC[a] clinical stage C	0.98 (0.43–2.21)	0.96

Univariate analyses. Significant *P* values are in bold. CI, confidence interval.
[a]Centre for Disease Control.

subsets, those that are associated with the highest level of γ-IFN production, a major cytokine involved in the defence against *T. gondii*. The production of γ-IFN was also strongly correlated with lymphoproliferative responses to *T. gondii* in our study. Furthermore, we have also shown that the increase in the number of terminally differentiated CD8 T cells was strongly associated with the production of γ-IFN in the presence of *T. gondii* antigens (Table 4). The use of experimental models has indeed provided evidence that γ-IFN production by CD8 T cells induces immunity to *T. gondii* infection and that these cells probably act additively or synergistically with CD4 T cells to prevent the reactivation of chronic *T. gondii* infection, with CD8 subsets likely to play a more dominant role [17,22,31–33]. CD8-mediated cytotoxic immune responses to *T. gondii* have also been identified in humans [34].

Our results, therefore, suggest that monitoring of effector memory CD4 T cells and terminally differentiated CD8 T cells in patients on HAART might be a better marker of the restoration of *T. gondii*-specific immune responses than the total number of CD4 T cells. However, our study has some limitations: it is a relatively small study, and differences seen between HIV-infected patients and controls might be also explained, at least in part, by demographic differences, that is controls were younger and more frequently female. Also, we could not establish *T. gondii*-specific lymphocyte proliferation responses as the cause of protection against toxoplasmosis, and there is

Table 4. Univariable analyses of time-dependent predictive factors of a positive lymphoproliferative response and γ-IFN production to *Toxoplasma* antigen among HIV-infected patients.

	Lymphoproliferative response			γ-IFN production		
	OR of response (95% CI)		*P*	OR of response (95% CI)		*P*
Viral load (log_{10} copies)	1.1873	(0.851–1.657)	0.31	0.8325	(0.5760–1.2033)	0.33
Lymphocytes	1.0007	(0.999–1.001)	0.09	1.0009	(1.0003–1.0016)	**0.006**
CD4 cells	1.0044	(0.999–1.009)	0.06	1.0028	(0.9993–1.0064)	0.12
Central memory CD4 cells	1.0023	(0.992–1.013)	0.66	1.0004	(1.0073–1.0080)	0.92
Effector memory CD4 cells	1.0199	(1.004–1.036)	**0.02**	1.0160	(1.0036–1.0284)	**0.011**
Terminally differentiated	1.0316	(0.961–1.108)	0.39	1.0333	(0.9881–1.0800)	0.15
Naive CD4 cells	1.0058	(0.998–1.014)	0.17	1.0033	(0.9952–1.0111)	0.43
Competent CD4 cells	1.0036	(0.999–1.008)	0.14	1.0021	(0.9983–1.0058)	0.28
CD8 cells (cells/μl)	0.9994	(0.999–1.002)	0.30	1.0012	(1.0002–1.0022)	0.017
Central memory CD8 cells	1.0038	(0.988–1.020)	0.65	1.0025	(0.9871–1.0182)	0.76
Effector memory CD8 cells	1.0009	(0.998–1.003)	0.46	1.0016	(0.9995–1.0030)	0.13
Terminally differentiated CD8 cells	1.0009	(0.999–1.003)	0.40	1.0022	(1.0006–1.0030)	**0.008**
Naive CD8 cells	1.0044	(0.997–1.012)	0.27	1.0057	(1.0001–1.0111)	0.046
Competent CD8 cells	1.0018	(0.999–1.005)	0.21	1.0025	(1.0000–1.0050)	0.051
B cells	0.9998	(0.994–1.005)	0.94	0.9991	(0.9946–1.0036)	0.69

Significant *P* values are in bold. CI, confidence interval; OR, odds ratio.

the possibility that some other factors such as cytotoxic responses that were not assayed in this study might correlate better.

In conclusion, our study showed that in-vitro specific immune reconstitution against *T. gondii* emerged in most HIV-infected patients with a low baseline CD4 cell count after 12 months of HAART. However, a significant proportion of these patients did not exhibit a normal lymphoproliferative response or γ-IFN production to *Toxoplasma* antigen. In these patients, the assessment of the effector memory CD4 and terminally differentiated CD8 T-cell subsets could be a better marker of immune reconstitution than the total CD4 cell count. These data will also help to better understand the determinants of the restoration of *T. gondii*-specific immune responses in patients with advanced HIV-infection starting HAART.

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Authors' contribution to the study: A.F. and J.-M.M. designed the study and wrote the protocol. A.F., J.P., J.-M.M.M. and N.D.C. managed the patients and collected the clinical data. M.C., C.R. and D.C. performed the immunological tests and collected the immunological data. Y.J.-F.G. and F.D. performed the western blots for antitoxoplasma antibodies. S.C. analysed the data with the contribution of all authors. J.-M.M.M. wrote the first draft of the manuscript that was critically revised by all authors.

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References

1. Palella FJ, Delaney KM, Moorman AC, Loveless MO, Fuhrer J, Satten GA, *et al*. **Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection.** *N Engl J Med* 1998; **338**:853–860.
2. Mocroft A, Vella S, Benfield TL, Chiesi A, Miller V, Gargalianos P, *et al*. **Changing patterns of mortality across Europe in patients infected with HIV-1. EuroSIDA study group.** *Lancet* 1998; **352**:1725–1730.
3. Li T, Tubiana R, Katlama C, Calvez V, Ait Mohand H, Autran B. **Long-lasting recovery in CD4 T-cell function and viral-load reduction after highly active antiretroviral therapy in advanced HIV-1 disease.** *Lancet* 1998; **351**:1682–1686.
4. Komanduri KV, Viswanathan MN, Wieder ED, Schmidt DK, Bredt BM, Jacobson MA, McCune JM. **Restoration of cytomegalovirus-specific CD4+ T-lymphocyte responses after ganciclovir and highly active antiretroviral therapy in individuals infected with HIV-1.** *Nat Med* 1998; **4**:953–956.
5. Wendland T, Furrer H, Vernazza P, Frutig K, Christen A, Matter L, *et al*. **HAART in HIV-infected patients: restoration of antigen-specific CD4 T-cell responses *in vitro* is correlated with CD4 memory T-cell reconstitution, whereas improvement in delayed type hypersensitivity is related to a decrease in viraemia.** *AIDS* 1999; **13**:1857–1862.
6. Autran B, Garcelain G, Li TS, Blanc C, Mathez D, Tubiana R, *et al*. **Positive effects of combined antiretroviral therapy on CD4+ T cell homeostasis and function in advanced HIV disease.** *Science* 1997; **277**:112–116.
7. Fournier S, Rabian C, Alberti C, Carmagnat MV, Garin JF, Charron D, *et al*. **Immune recovery under highly active antiretroviral therapy is associated with restoration of lymphocyte proliferation and gamma-interferon production in presence of *Toxoplasma gondii* antigens.** *J Infect Dis* 2001; **183**:1586–1591.
8. Furrer H, Opravil M, Bernasconi E, Telenti A, Egger M, for the Swiss HIV cohort study. **Stopping primary prophylaxis in HIV-1-infected patients at high risk of toxoplasma encephalitis.** *Lancet* 2000; **355**:2217–2218.
9. Mussini C, Pezzotti P, Govoni A, Borghi V, Antinori A, D'Arminio Monforte A, *et al*. **Discontinuation of primary prophylaxis for *Pneumocystis carinii* pneumonia and toxoplasmic encephalitis in human immunodeficiency virus type I-infected patients: the changes in opportunistic prophylaxis study.** *J Infect Dis* 2000; **181**:1635–1642.
10. Zeller V, Truffot C, Agher R, Bossi P, Tubiana R, Caumes E, *et al*. **Discontinuation of secondary prophylaxis against disseminated *Mycobacterium avium* complex infection and toxoplasmic encephalitis.** *Clin Infect Dis* 2002; **34**:662–667.
11. Miro JM, Lopez JC, Podzamczer D, Pena JM, Alberdi JC, Martinez E, *et al*. **Discontinuation of primary and secondary *Toxoplasma gondii* prophylaxis is safe in HIV-1 infected patients after immunological restoration with highly active antiretroviral therapy: results of an open, randomized, multicenter clinical trial.** *Clin Infect Dis* 2006; **43**:79–89.
12. US Public Health Service (USPHS) and Infectious Disease Society of America (IDSA). **2002 USPHS/IDSA guidelines for preventing opportunistic infections among HIV-infected persons.** *MMWR Recomm Rep* 2002; **51**:1–46.
13. Benson CA, Kaplan JE, Masur H, Pau A, Holmes KK. **Treating opportunistic infections among HIV-infected adults and adolescents: recommendations from CDC, the National Institutes of Health, and the HIV Medicine Association/Infectious Diseases Society of America.** *Clin Infect Dis* 2005; **40**:S131–S235.
14. Derouin F, Rabian-Herzog C, Ballet J. **Impaired *in vitro* lymphocyte response to Toxoplasma antigen in HIV-1 infected patients.** *J Clin Lab Immunol* 1989; **28**:179–182.
15. Miro JM, Lejeune M, Claramonte X, Martinez E, Ribera E, Arrizabalaga J, *et al*. **Timing of renconstitution of *Toxoplasma gondii*-specific T-cell responses in AIDS patients with acute toxoplasmic encephalitis after starting HAART. A prospective multicenter longitudinal study** [abstract 796]. In: *Program and abstracts of the 10th Conference on Retroviruses and Opportunistic Infections* (Boston); 2003.
16. Alfonzo M, Blanc D, Troadec C, Eliaszewicz M, Gonzalez G, Scott-Algara D, *et al*. **Temporary restoration of immune response against *Toxoplasma gondii* in HIV-infected individuals after HAART, as studied in the hu-PBMC-SCID mouse model.** *Clin Exp Immunol* 2002; **129**:411–419.
17. Gazzinelli R, Xu Y, Hieny S, Cheever A, Sher A. **Simultaneous depletion of CD4+ and CD8+ T lymphocytes is required to reactivate chronic infection with *Toxoplasma gondii*.** *J Immunol* 1992; **149**:175–180.
18. Belanger F, Derouin F, Grangeot-Keros L, Meyer L, and the HEMOCO and SEROCO study group. **Incidence and risk factors of toxoplasmosis in a cohort of human immunodeficiency virus-infected patients: 1988–1995.** *Clin Infect Dis* 1999; **28**:575–581.

19. Luft BJ, Hafner R, Korzun AH, Leport C, Antoniskis D, Bosler EM, *et al*. **Toxoplasmic encephalitis in patients with the acquired immunodeficiency syndrome.** *N Engl J Med* 1993; **329**:995–1000.
20. Murray HW, Hillman JK, Rubin BY, Kelly CD, Jacobs JL, Tyler LW, *et al*. **Patients at risk for AIDS-related opportunistic infections. Clinical manifestations and impaired gamma interferon production.** *N Engl J Med* 1985; **313**:1504–1510.
21. Suzuki Y, Orellana M, Schreiber R, Remington J. **Interferon-gamma: the major mediator of resistance against *Toxoplasma gondii*.** *Science* 1988; **240**:516–518.
22. Beaman M, Wong S, Remington J. **Cytokines, *toxoplasma* and intracellular parasitism.** *Immunol Rev* 1992; **127**:97–117.
23. Ullum H, Cozzi Lepri A, Bendtzen K, Victor J, Gotzsche PC, Phillips AN, *et al*. **Low production of interferon gamma is related to disease progression in HIV infection: evidence from a cohort of 347 HIV-infected individuals.** *AIDS Res Hum Retroviruses* 1997; **13**:1039–1044.
24. Derouin F, Leport C, Pueyo S, Morlat P, Letrillart B, Chene G, *et al*. **Predictive value of *Toxoplasma gondii* antibody titres on the occurrence of toxoplasmic encephalitis in HIV-infected patients.** *AIDS* 1996; **10**:1521–1527.
25. Sieg SF, Mitchem JB, Bazdar DA, Lederman MM. **Close link between CD4+ and CD8+ T cell proliferation defects in patients with human immunodeficiency virus disease and relationship to extended periods of CD4+ lymphopenia.** *J Infect Dis* 2002; **185**:1401–1408.
26. Lederman HM, Williams PL, Wu J, McCutchan JA, Cohn SE, Murphy RL, *et al*. **Incomplete immune reconstitution after initiation of highly active antiretroviral therapy in human immunodeficiency virus-infected patients with severe CD4+ cell depletion.** *J Infect Dis* 2003; **188**:1794–1803.
27. Komanduri KV, Feinberg J, Hutchins RK, Frame RD, Schmidt DK, Viswanathan MN, *et al*. **Loss of cytomegalovirus-specific responses in human immunodeficiency virus type 1-infected patients with high CD4+ T-cell counts and recurrent retinitis.** *J Infect Dis* 2001; **183**:1285–1289.
28. Lilleri D, Piccinini G, Baldanti F, Seminari E, Galloni D, Gerna G. **Multiple relapses of human cytomegalovirus retinitis during HAART in an AIDs patient with reconstitution of CD4+ T-cell count in the absence of HCMV-specific CD4+ T-cell rsponse.** *J Clin Virol* 2003; **26**:95–100.
29. French MA, Keane M, McKinnon EJ, Phung S, Price P. **Susceptibility to opportunistic infections in HIV-infected patients with increased CD4 T-cell counts on antiretroviral therapy may be predicted by markers of dyfunctional effector memory CD4 T cells and B cells.** *HIV Med* 2007; **8**:148–155.
30. Connick E, Lederman MM, Kotzin BL, Spritzler J, Kuritzkes DR, St Clair M, *et al*. **Immune reconstitution in the first year of potent antiretroviral therapy and its relationship to virologic response.** *J Infect Dis* 2000; **181**:358–363.
31. Khan IA, Kasper LH. **IL-15 augments CD8+ T cell mediated immunity against *Toxoplasma gondii* infection in mice.** *J Immunol* 1996; **157**:2103–2118.
32. Gazzinelli RT, Hakim FT, Hieny S, Shearer GM, Sher A. **Synergistic role of CD4+ and CD8+ lymphocytes in IFN-gamma production and protective immunity induced by an attenuated *Toxoplasma gondii* vaccine.** *J Immunol* 1991; **146**:286–292.
33. Parker SJ, Roberts CW, Alexander J. **CD8+ T cells are the major lymphocyte subpopulation involved in the protective immune response to *Toxoplasma gondii* in mice.** *Clin Exp Immunol* 1991; **84**:207–212.
34. Purner MB, Berens RL, Nash PB, Van Linden A, Ross E, Kruse C. **CD4-mediated and CD8-mediated cytotoxic and proliferative immune responses to *Toxoplasma gondii* in seropositive humans.** *Infect Immun* 1996; **64**:4330–4338.