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Polyclonal serum-free light chains elevation in HIV-infected patients

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We investigated the association between polyclonal serum-free light chains and prognostic biomarkers routinely used in the setting of HIV infection. For this purpose serum samples of 182 HIV-infected patients from the Italian Cohort of Antiretroviral Naive Patients foundation cohort were analysed. We found that polyclonal serum free light chains above the upper normal limit are strongly correlated in HIV-infected patients with advancing age, shorter time of undetectable HIV viremia, higher viral load and with lower CD4 cell count at sample.

Combination antiretroviral therapy (cART) has greatly reduced the morbidity and mortality of HIV-infected patients [1]. However, despite long-term suppression of HIV-RNA and $CD4^+$ T-cell reconstitution in most patients following cART, full life expectancy is restored in some, but not all, patients; thus, HIV-infected individuals experience high rates of cardiovascular disease, non-AIDS malignancies, bone, renal and liver disorders and dementia [2,3]. Factors that drive ongoing morbidity include a variable combination of immune activation, premature aging of the immune system and cART toxicity [4].

Several large studies have shown that cell-associated and soluble markers of immune activation and inflammation and circulating microbial products are elevated in HIV-infected patients undergoing cART and consequently associated with adverse clinical outcomes; however, very few studies focused on chronic B-cell dysfunction soluble biomarkers [5].

Recently increased polyclonal serum-free light chains (sFLCs) have been detected in several conditions characterized by chronic B-cell activation/dysfunction [6–8]. Furthermore, polyclonal elevation of sFLCs was shown to be a strong risk factor of non-Hodgkin lymphoma and Hodgkin lymphoma in HIV-infected patients [9,10].

The primary objective of this study was first to determine the polyclonal sFLC level in HIV-infected patients and then to evaluate their correlations with prognostic biomarkers routinely used in the setting of HIV infection.

For this analysis, 182 patients were enrolled in the study, belonging to the whole control cohort screened in our previous sFLCs lymphoma study [10]. In this study, the control population (now our study population) was chosen randomly among the members of the cohort, according to the statistical procedure for the design of a case–control study nested in a cohort study. Thus, controls (now our case patients) were selected as cases in this new analysis if 18 years or older, HIV infected and with available serum or plasma samples stored and no confounding comorbidities for the sFLCs elevation such as concomitant malignancies, monoclonal gammopathy of undetermined significance, renal impairment, autoimmune conditions like active rheumatic disease and other chronic inflammatory conditions.

For all patients, serum or plasma stored at $-80^{\circ}C$ were thawed and analyzed at the National Institute for Infectious Disease 'Lazzaro Spallanzani' laboratory. The sFLCs concentrations were determined using the quantitative FLC assay (FREELITE; The Binding Site, Birmingham, UK). The assay separately measure κ sFLC (normal range, 0.33–1.94 mg/dl) and λ sFLC (normal range, 0.57–2.63 mg/dl), and $\kappa:\lambda$ FLC ratio (normal range 0.26–1.65) [11,12]. Because the polyclonal nature of sFLCs was the focus of our study, we introduced the $k+\lambda$ sum (normal range, 0,59–4,57 mg/dl) as the measurement of choice and as the primary variable studied.

The purpose of our analysis was to determine the association between polyclonal sFLCs concentration and routinely used HIV biomarkers. Wilcoxon test was used to compare FLC $k+\lambda$ value between different risk categories. We then investigated the univariable and multivariable associations between sFLCs value over the upper normal limit (UNL) and the following variables: sex, age, mode of HIV transmission, nadir of CD4 cell count, CD4 cell count at sample time, $\log_{10}$ HIV-RNA at sample time, time spent with an undetectable viral load prior to date of sample, HAART exposure at sample time, Center for Disease Control stage, Hepatitis C Virus (HCV) status, calendar year of sample date, hemoglobin, white blood cell count, platelet count. Univariable and multivariable analysis were performed using a logistic regression model. Statistical analysis was performed using STATA statistical software, release 10 (1999), (Stata Corporation, College Station, Texas, USA)

One hundred and eighty-two patients' plasma samples were analyzed. The main characteristics of the study population have been described and summarized in detail in Table 1. Aiming to identify factors associated with different levels of polyclonal sFLCs, it is interesting to note that even if nearly all the possible confounding comorbidities for the sFLCs elevation were excluded, patients had sFLC higher than the UNL value of the test [k + λ, mg/dl; median Interquartile range = 5.98 (4.22–9.89)]. Further, the k : λ ratio was found normal in all samples, confirming the polyclonal nature of the B-cell population synthesizing the excess of sFLCs.

The key question of this study was to investigate whether polyclonal sFLC sum was associated with prognostic biomarkers routinely used in the setting of HIV infection. Comparing k + λ value in different groups, patients with shorter time with suppressed HIV viremia (<1 vs. >1 year), higher viral load at sample (>500 vs. <500 copies/ml) and lower CD4 cell count (0–499 vs.

Table 1. Demographic and clinical characteristic of the 182 HIV-infected patients studied.

Characteristics	Patients $N = 182$ N° (%)	k + λ, mg/dl median (IQR)	*P*	Univariate analysis			Multivariable analysis		
				OR	95% CI	*P*	AOR	95% CI	*P*
Age years, median (IQR)									
<40 years	54 (29.7)	6.12 (4.14–9.20)	0.858						
≥40 years	128 (70.3)	5.88 (4.25–10.41)							
Per 10 years older[a]				0.86[a]	0.58–1.25	0.422	1.95[a]	1.02–3.71	0.044
Male sex									
F	21 (11.5)	6.26 (4.99–7.54)	0.674	1			1		
M	161 (88.5)	5.82 (4.10–10.45)		0.52	0.17–1.63	0.264	0.29	0.05–1.75	0.177
Mode of HIV transmission									
Heterosexual	49 (26.9)	5.94 (3.49–11.64)	0.0006	1			1		
IVDU	66 (36.3)	7.82 (5.33–12.56)		3.22	1.34–7.76	0.009	2.21	0.40–12.35	0.366
Homosexual	52 (28.6)	5.14 (3.80–6.48)		0.86	0.27–2.80	0.807	1.30	0.15–11.61	0.812
Other	15 (8.2)	6.62 (3.49–11.64)		0.91	0.41–2.03	0.817	0.50	0.15–1.67	0.260
HCV status									
Negative	102 (56.0)	5.10 (3.79–7.01)	0.0001	1			1		
Positive	80 (44.0)	7.07 (5.29–11.96)		3.46	1.70–7.07	0.001	3.82	0.75–19.35	0.105
CDC stage									
A/B	157 (86.3)	5.81 (4.10–9.75)	0.219	1			1		
C	25 (13.7)	6.26 (4.81–12.29)		1.39	0.52–3.71	0.505	0.78	0.17–3.64	0.752
On HAART at sample									
Yes	111 (61.0)	5.12 (3.79–6.76)	0.0001	1			1		
No	71 (39.0)	8.61 (5.54–13.75)		0.29	0.14–0.61	0.001	2.30	0.64–8.23	0.200
$CD4^{+}$ cells/μl									
0–499	74 (40.7)	7.06 (5.24–12.94)	0.0001	1			1		
≥500	108 (59.3)	5.24 (3.87–7.29)		0.75[a]	0.67–0.85	0.0001	0.68[a]	0.54–0.87	0.002
Per 100 cell higher[a]									
HIV-RNA									
<500 copies/ml	79 (43.4)	4.87 (3.78–6.43)	0.0001	1			1		
≥500 copies/ml	103 (56.6)	8.20 (5.68–12.56)		2.09[a]	1.47–2.96	0.0001	2.12[a]	1.14–3.93	0.017
Per 1 $\log_{10}$ higher[a]									
Years of viral suppression									
≤1	86 (47.3)	8.01 (5.35–12.86)	0.0001	1			1		
>1	96 (52.7)	4.87 (3.78–6.61)		0.73[a]	0.63–0.85	0.0001	0.62[a]	0.44–0.86	0.005
Per 1 years higher[a]									
Calendar years of sample									
≤2002	91 (50.0)	6.17 (4.22–11.11)	0.415	1			1		
>2002	91 (50.0)	5.68 (4.14–9.20)		0.93[a]	0.83–1.05	0.232	1.12[a]	0.91–1.39	0.294
1 year more[a]									
WBC $\times 10^{9}$/l									
≥1.0	138 (92.0)	5.89 (4.14–10.38)	0.233	1			1		
<1.0	12 (8.0)	4.83 (3.90–6.56)		0.77[a]	0.65–0.91	0.002	0.90[a]	0.76–1.08	0.257
Per 10^{6} higher[a]									
Hemoglobin (g/dl)									
≤14.5	74 (49.3)	6.77 (4.59–12.06)	0.006	1			1		
>14.5	76 (50.7)	5.33 (3.83–7.01)		0.75[a]	0.57–0.99	0.042	0.86[a]	0.59–1.26	0.444
Per 1 g/dl higher[a]									
Platelets $\times 10^{9}$/l									
≤100	15 (10.0)	11.49 (6.04–16.54)	0.004	1			1		
>100	135 (90.0)	5.60 (4.07–8.61)		0.94[a]	0.90–0.98	0.010	1.05[a]	0.97–1.14	0.210
Per 10^{3} higher[a]									

AOR, adjusted odd ratio; CDC, Center for Disease Control; CI, confidence interval; HCV, hepatitis C virus; IVDU, intravenous drug user; IQR, interquartile range; OR, odds ratio; sFLCs, serum free light chains. Main k + λ sFLCs levels according to the variable studied. k + λ normal value = 0.59–4.57 mg/dl.

[a]Univariate and multivariable analysis (From the left to right of the table).

>500 cells) presented statistically significant higher k + λ sFLC. Regarding therapy, untreated patients have a significantly higher k + λ sFLC value compared to patients who were in treatment with HAART. Not surprisingly, and as previously reported, patients with HCV coinfection had significantly higher levels of polyclonal sFLCs.

At multivariate logistic regression, analysis of only age [odd ratio (OR) per 10 years older 1.95 $P = 0.044$], $CD4^+$ cell count (OR 0.68 per 100 cells higher $P = 0.002$), HIV-RNA (OR 2.12 per 1 log higher $P = 0.017$) and years of viral suppression (OR 0.62 per 1 year higher $P = 0.005$) were found to be independently associated with polyclonal sFLCs above the UNL (Table 1).

The major finding of our research is that elevated polyclonal sFLCs, a marker of B-cell expansion/dysfunction, strongly correlate in HIV-infected patients with advancing age, shorter time of undetectable HIV viremia, higher viral load and with lower CD4 cell count at sample. This finding was substantially strengthened by the absence of confounding comorbidities for the sFLCs elevation in the inclusion criteria of patients. To our knowledge, this is the first time that an association between polyclonal sFLCs and the other prognostic biomarkers routinely used in the setting of HIV infection has been reported.

In conclusion, as this biomarker is detected with a simple blood test, quantifying polyclonal sFLCs in HIV-infected individuals may represent a significant marker of immune activation and of unresolved inflammation, allowing us to better identify patients on cART who are at risk of immune dysfunction and non-AIDS events. However, further studies performed on a larger number of samples are needed to confirm and expand our findings.

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Conflicts of interest

There are no conflicts of interest.

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Thalidomide for steroid-dependent immune reconstitution inflammatory syndromes during AIDS

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Management of relapsing or refractory immune reconstitution inflammatory syndromes (IRISs) despite corticosteroid therapy is yet to be defined. We describe three HIV-infected patients with corticosteroid-dependent and life-threatening paradoxical immune reconstitution inflammatory syndrome for whom thalidomide treatment induced rapid clinical remission and permitted complete corticosteroid withdrawal without clinical relapse.

HAART in advanced phases of HIV-1 infection may induce immune reconstitution inflammatory syndrome (IRIS), requiring brief corticosteroid therapy in severe cases [1,2]. Clinical manifestations in 'paradoxal IRIS' are primarily a worsening of clinical signs or the appearance of new events in patients with a known infection after starting HAART, most often without detectable or viable opportunistic infection and despite a successful suppression of HIV plasma viremia [3]. More rarely, in a form called 'unmasking IRIS', in which the opportunistic infection preexisted but was clinically silent, symptoms develop after the introduction of HAART. A switch from a type 2 helper T cell (Th2) to a Th1 immune response after HAART introduction is suspected, as suggested by increased levels of interferon gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) in tuberculosis-associated IRIS [4], and the successful use of monoclonal TNF-α antagonist antibodies [5,6]. Thalidomide has shown in-vitro inhibition of TNF-α production [7], benefit during experimental tuberculosis meningitis [8], and response in a case of cryptococcal-associated IRIS [9].

We have report three cases of HIV-infected patients with refractory, corticosteroid-dependent and life-threatening paradoxal IRIS for whom thalidomide administration resulted in rapid clinical remission. The first case is a 33-year-old HIV-infected man who was admitted in April 2008 with cryptococcal meningitis. The CD4 cell count was 15 cells/μl and HIV-1 plasma viral load was 96 600 copies/ml. The clinical course was favorable after antifungal therapy and introduction of HAART at day 28. In September 2009, the patient presented symptoms of meningitis and cerebellar ataxia despite fluconazole prophylaxis (400 mg per day), undetectable plasma viral load and a CD4 cell count of 273 cells/μl. An extensive infectious work-up analysis revealed no active infection, an increased cerebral spinal fluid (CSF) opening pressure and CSF encapsulated yeast-like fungi with sterile cultures. Cerebral MRI results are shown in Fig. 1a. Paradoxal IRIS was suspected and intravenous (i.v.) methylprednisolone 250 mg per day was administered for 3 days, followed by oral prednisolone (1 mg/kg per day) with progressive tapering to 15 mg per day in the following 8 weeks. Fluconazole (800 mg per day) was continued. The patient presented three other IRIS relapses uncovered by cephalalgia and diplopia when corticosteroids were tapered to a daily dose of 12.5–15 mg. Thalidomide was initiated at 100 mg per day with aspirin 75 mg per day in August 2010, allowing complete cessation of corticosteroids in March 2012 with no following relapse (Fig. 1b).

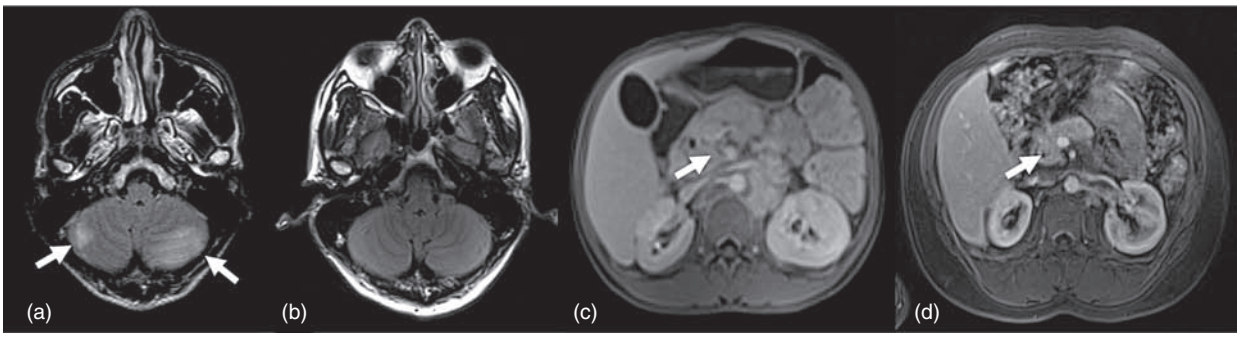

Fig. 1. Axial cerebral MRI in fluid-attenuation inversion recovery sequences (case 1) and axial abdominal MRI (case 3) in T2-weighted sequences following the diagnosis of a relapse immune reconstitution inflammatory syndrome and with treatment (steroid and thalidomide). (a) Bilateral cerebellar hyperintensity in September 2009 (arrows). (b) Disappearance of cerebellar hyperintensity in October 2011 after 14 months of thalidomide (100 mg per day). (c) Voluminous mass encircling the mesenteric vessels in August 2010 (arrow). (d) Major decrease in the size of abdominal mass in January 2011 after 3 months of thalidomide (100 mg per day) (arrow). FLAIR, fluid-attenuation inversion recovery; IRIS, immune reconstitution inflammatory syndrome.

The second case was a 47-year-old man who was admitted in December 2004 with cryptococcal meningitis and HIV infection (CD4 cell count was 19 cells/μl and HIV-1 plasma viral load was 462 292 copies/ml). Initial evolution was favorable for antifungal therapy and introduction of HAART at day 21 and fluconazole maintenance therapy (400 mg per day). In December 2006, he was admitted with encephalitis. HIV viral load was undetectable and CD4 cell count was 353 cells/μl. An extensive infectious work-up analysis of active infections, including a neurosurgical biopsy of right parietal-frontal meninges, was negative. Cerebral MRI showed right parietal-temporal hyperintensity in fluid-attenuation inversion recovery sequences and T1-weighted subarachnoid space enhancement intensity with gadolinium. Paradoxal IRIS was suspected and high dose of i.v. methylprednisolone (500 mg per day) was administered for 3 days followed by oral prednisolone (1 mg/kg per day) progressively tapered, in association with fluconazole (400 mg per day). Corticosteroids and fluconazole were interrupted in January and March 2009 respectively. In May 2009, the patient presented with a first relapse of IRIS treated with prednisone (60 mg per day) and fluconazole (400 mg per day). Two successive episodes, despite 15 mg per day of prednisolone, justified thalidomide introduction (100 mg per day) with aspirin in December 2010. In June and October 2011, prednisone and fluconazole were successfully interrupted. Thalidomide was interrupted in March 2012 with no relapse at the time of writing (June 2012).

In December 2007, the third case, a 19-year-old HIV-infected girl was diagnosed with disseminated tuberculosis in the context of immunovirologic failure despite HAART (CD4 cell count was 15 cells/μl and HIV-1 plasma viral load was 259 327 copies/ml). Antimycobacterial therapy was introduced and HAART optimized. Seven months later, she was admitted for abdominal pain and acute renal insufficiency (CD4 cell count of 104 cells/μl and HIV viral load of 567 copies/ml). Renal biopsies revealed gigantocellular and epithelioid granuloma without caseous necrosis. Cultures were negative. Paradoxal IRIS was suspected, and high doses of prednisone were introduced (2 mg/kg per day for 3 days, progressively tapered), resulting in rapid biological and clinical improvement at day 9. However, the patient presented two relapses of IRIS despite tuberculosis therapy after 4 and 23 months, the first when prednisone was tapered to 20 mg per day and the second 1 month after corticosteroid interruption, with intensive, transfixing abdominal pain linked to mesenteric necrotic lymph nodes (Fig. 1c). Thalidomide was introduced (100 mg per day) with prednisone (40 mg per day), aspirin and effective contraception measures, inducing rapid clinical and MRI responses (Fig. 1d). Prednisone and thalidomide were respectively interrupted 10 and 14 months later, with no sign of relapse in June 2012.

We retrospectively quantified the cytokine concentrations of TNF-α, eotaxin, MIP-1β, MIG, IL-15, IL-17, IL-1RA, IL-2R, GM-CSF, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, MCP-1, MIP-1α, RANTES, IP-10, IFN-γ and IFN-α in serum using a multiplexed microbead assay (cytokine human 25 plex Panel, Life Technologies, Saint Aubin, France) and a Luminex 100 apparatus (Luminex, Oosterhout, The Netherlands) during and after introduction of thalidomide. The local ethical committee approved the protocol and consent was obtained from all patients. No specific pattern of cytokine concentration and evolution could be identified.

We have described three cases of HIV-infected patients with severe and corticosteroid-dependent IRIS for whom thalidomide allowed steroid tapering. Both the underlying physiopathological immune mechanisms and the analogy with tuberculosis-related IRIS justified the initial administration of high-dose methylprednisolone. Rapid clinical remission followed steroid therapy, though clinical remissions were steroid dose-dependent in both cases of cryptoccocal-related IRIS. Thalidomide has been used in other IRIS-like syndromes such as treatment of erythema nodosum leprosum [7]. Thalidomide (2 mg/kg per day) seemed beneficial in two HIV-infected children in a case series of four tuberculous meningitis complicated by IRIS [10]. However, one randomized trial of thalidomide in childhood tuberculous meningitis had to be interrupted prematurely due to important adverse events in the thalidomide arm [11], but thalidomide doses were very important (24 mg/kg per day), and thalidomide was introduced after randomization independently of IRIS presence. In our cases, clinical responses were rapid under thalidomide and cortisone therapy and were maintained after tapering of corticosteroids, arguing for a direct and strong anti-inflammatory effect of thalidomide. The drug was also well tolerated at low doses, with only mild dizziness and constipation reported in two patients. No clear immunomodulatory effect of thalidomide on cytokine levels could be observed. Similarly, the effect of thalidomide on TNF-α synthesis was not confirmed in two randomized clinical trials of oral aphtous ulcers and Kaposi sarcoma in HIV-infected patients treated with thalidomide or placebo [12,13].

In conclusion, thalidomide may be an interesting salvage treatment in patients with cortico-dependent or refractory IRIS, and should be further studied in clinical trials.

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Conflicts of interest

There are no conflicts of interest.

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Identification of HIV-1-specific regulatory T-cells using HLA class II tetramers

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Regulatory T cells (Tregs) are potent immune modulators, but their precise role in HIV pathogenesis remains incompletely understood. Most studies to date have focused on frequencies or phenotypes of 'bulk' Treg populations. However, although antigen-specific Tregs have been reported in other diseases, HIV-1 epitope-specific Tregs have not been described to date. We here report the first identification of functional HIV-1-Gag-specific regulatory T cells using human leukocyte antigen class II tetramer staining in HIV-1-infected individuals.

Regulatory T cells (Tregs) are potent immune modulators and serve an important function in human immune homeostasis. Despite an increasing body of data on regulatory T cells in HIV-1 infection, their role in HIV-1 pathogenesis remains inadequately understood. Although some data argue in favor of a beneficial effect of Tregs through impact on HIV-1-associated immune activation [1] and more recently viral replication [2], other data support a deleterious effect by suppressing critical virus-specific immune responses [3,4]. Controversy also remains about the fate of regulatory T cells during progressive HIV-1 infection, with some studies reporting declining Treg numbers and other studies demonstrating increased Treg frequencies [3,5,6]. Although 'bulk' Treg populations have been studied extensively in recent years in the context of HIV-1 infection, no reliable data is available on HIV-1 specificity of regulatory T cells and whether these cells are induced in infected individuals.

Part of the challenges in detecting antigen-specific Treg populations relate to the limited availability of direct visualization tools such as human leukocyte antigen (HLA) class II tetramers. Another barrier to the study of HIV-1-specific Tregs lies in the paucity of even 'bulk' Treg populations in HIV-1-infected individuals. We and others recently reported median frequencies of $CD4^+CD25^+CD127^-FoxP3^+$ regulatory T cells of

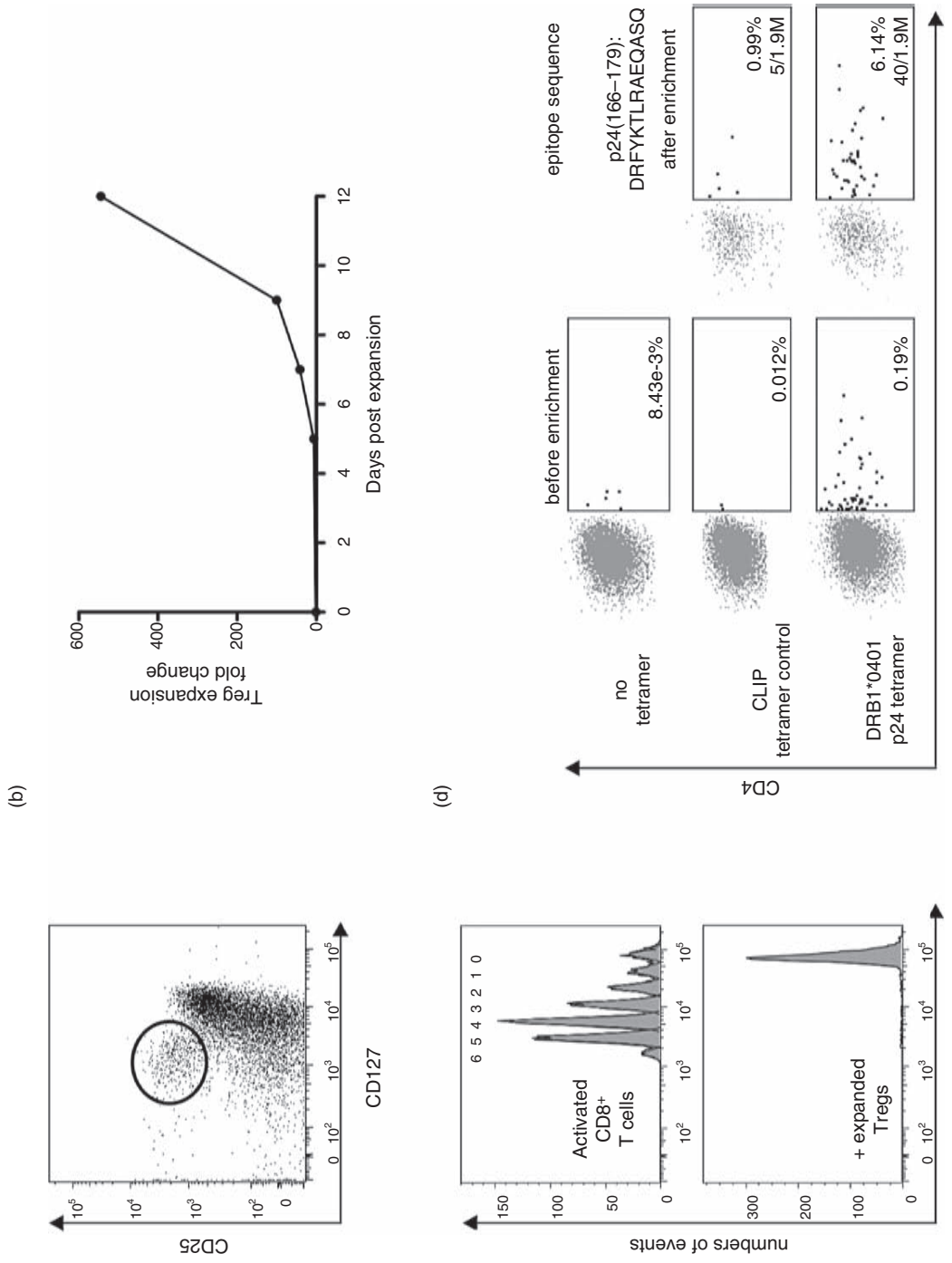

Fig. 1. (a) Representative example of CD4$^+$ regulatory T-cell (Treg) staining by flow cytometry with gating strategy before flow-based sorting and Treg expansion. Tregs are defined as CD25$^+$CD127^{low} CD4$^+$ T cells. (b) Mean expansion fold change of the expanded regulatory T-cell lines that were stained with HIV-1-p24-gag-specific HLA class II tetramer. (c) Representative histogram plots showing T-cell proliferation by CFSE dilution of CD8$^+$ T cells after 4 days of culture following stimulation with anti-CD3/CD2/CD28-coated beads, cocultured with (lower histogram) or without expanded Tregs (upper histogram) at a ratio of 1:1 Treg per PBMC. (d) Example of PE-conjugated-HLA class II tetramer staining on expanded Tregs isolated from an individual with untreated chronic progressive HIV-1 infection before and after PE-enrichment over a magnetic column. The cells were incubated alone (upper dot plot), in presence of an HLA-DR0401-restricted HLA class II tetramer loaded with a control CLIP peptide (middle dot plots) or the p24-Gag peptide DRFYKTLRAEQASQ (lower dot plots). Percentages refer to tetramer positive cells per total CD4$^+$ T cells. Equal numbers of input cells were used for all staining and enrichment procedures.

4.5–7% (range 0.99–13.1%) of the $CD4^+$ T-cell population in untreated infected individuals, with absolute Treg numbers declining over time during disease progression [4,6,7], further complicating the detection of small HIV-1-epitope-specific Treg subpopulations *ex vivo*.

Antigen-specific Tregs have been reported and successfully visualized by HLA class II tetramers in murine and human studies of transplantation [8], diabetes, influenza [9] and autoimmunity [10,11]. In order to screen for HIV-1-specific regulatory T-cell populations, we first flow-sorted (gating scheme represented in Fig. 1a) and expanded Tregs *ex vivo* from eight HLA-DRB1*0401-expressing HIV-1-infected individuals (four individuals with chronic untreated progressive HIV-1 infection, three HIV elite controllers with undetectable HIV-1 viremia in the absence of therapy and one HAART-treated individual with undetectable HIV-1 viral load) using anti-CD3/anti-CD28-coated microbeads and IL-2 [12]. During the 12-day in vitro culture, Tregs expanded by a median of 580 fold (IQR 186 and 871) (Fig. 1b) and were tested for their suppressive capacity by standard carboxyfluorescein succinimidyl ester (CFSE) T-cell proliferation assays on day 7 using autologous bead-stimulated cryopreserved peripheral blood mononuclear cells (PBMCs) as responder cells. Expanded Tregs were highly suppressive (Fig. 1c), displayed the phenotype of 'activated' Tregs ($CD4^+CD45RA^-FoxP3^{hi}$) [13], and expressed high levels of classical Treg markers (HELIOS, CTLA4, FOXP3, CD39, CD25)(data not shown). Expanded Tregs were demethylated at the Treg-specific demethylation region locus of the *FOXP3* gene as evidenced by epigenetic analysis suggesting true origin from the regulatory T-cell lineage, as opposed to activation-induced transient FOXP3 upregulation [14]. We next stained the Treg lines with phycoerythrin (PE)-conjugated HLA class II tetramers specific for the HIV-p24-Gag epitope DRFYKTLRAEQASQ ($p24^{166–179}$). HLA-DR molecules with bound peptides from a self-protein, the invariant chain-derived CLIP peptide, were used as controls, as previously described [15]. Labeling with HIV-p24-Gag tetramers was considered positive when the T-cell population was more than three-fold larger compared to control tetramers, as defined in our previous studies using the same tetramer constructs for HIV-1 specific CD4 effector T-cell populations [16]. Two out of the eight HIV-1-positive study individuals with chronic untreated progressive HIV-1 infection had detectable responses to the p24-Gag class II tetramer at a frequency of 0.19 and 0.05% of CD4 in the nonenriched Treg culture, respectively. After tetramer-positive T-cell enrichment over a magnetic column, using anti-PE-conjugated magnetic beads [16], this frequency was enriched to 6.14 and 0.23% of Tregs, respectively (representative example shown in Fig. 1d). No tetramer-specific cells were demonstrated in HIV-1 negative control individuals or individuals lacking HLA-DRB1*0401 expression. These data demonstrate that HIV-1-epitope-specific Tregs can be detected in HIV-1 infected individuals using HLA class II tetramer technology.

To our knowledge this visualization by HIV-1-Gag-specific HLA class II tetramers represents the first identification of HIV-1-specific regulatory T cells reported to date. Although the epitope-specific Treg population was not readily detectable *ex vivo* from PBMCs, the magnitude of the tetramer-response after expansion was robust and comparable to frequencies for HLA class II-restricted responses in previous reports from other disease settings. Furthermore, higher frequencies of HIV-1-specific Tregs may be detectable using similar methods in lymphoid tissues such as the gut-associated lymphoid tissue in which increased frequencies of bulk Tregs have recently been described [4]. The specificity of the p24-Gag-epitope tested overlaps with a previously described HIV-1-specific $CD4^+$ effector T-cell response [16]. This finding is consistent with published data indicating that naive and memory $CD4^+$ Tregs can share the same T-cell receptor clonotypes as $CD4^+$ non-Treg in humans [17] and observations in murine models that regulatory and effector $CD4^+$ T cells may be driven by the same antigens [18]. Identification and further functional characterization of HIV-1-specific Tregs will also be important in HIV vaccine studies, in which vaccine strategies should be evaluated for their potential to induce not only HIV-1-specific effector populations, but also vaccine-induced HIV-1-specific regulatory T cells, which may negatively interfere with vaccine immunogenicity [19].

Taken together, these results show for the first time that HIV-1-specific regulatory T cells can be successfully visualized and isolated from HIV-1-infected individuals. The ability to identify and track HIV-1-specific regulatory T cells opens new opportunities to gain insight into the role of Tregs in HIV pathogenesis.

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the manuscript. M. Altfeld and B.D.W were involved in the establishment of the patient cohorts used and revised the manuscript. All authors have read and approved the text as submitted to AIDS.

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Conflicts of interest

The authors declare that they have no competing interests.

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