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HIV-Specific CD8$^+$ T Lymphocytes in Blood of Long-Term HIV-Infected Hemophilia Patients

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Abstract

Hemophilia patients infected with human immunodeficiency virus (HIV) 30 years ago show increased proportions of activated CD8$^+$DR$^+$ blood lymphocytes. We hypothesized that this might indicate a cellular immune response directed against HIV and might be the reason for long-term clinical stability of these patients. CD8$^+$ peripheral blood lymphocytes (PBL) reactive with six HIV and two cytomegalovirus (CMV) pentamers were determined in heparinized whole blood. Additional lymphocyte subsets as well as plasma cytokines and HIV-1 load were studied. Long-term HIV-infected hemophilia patients with ($n=15$) or without ($n=33$) currently detectable HIV-1 load in the plasma showed higher proportions of CD8$^+$ lymphocytes reactive with HIV ($p<0.001$) and CMV pentamers ($p=0.010$) than healthy individuals. The cellular anti-HIV response tended to be stronger and more polyclonal in patients during periods of viral replication than in patients with retroviral quiescence ($p=0.077$). Anti-HIV CD8$^+$ lymphocyte responses were strongest in patients with high counts of activated CD8$^+_{DR+}$ T ($r=0.353$; $p=0.014$) and low CD19$^+$ B lymphocyte counts ($r=-0.472$; $p=0.001$). Patients with or without HIV-1 viral load showed normal Th1 and Th2 plasma cytokine levels and high plasma interleukin-6 (versus healthy controls, $p=0.001$) and tumor necrosis factor-α ($p=0.020$). Hemophilia patients who have been living with HIV for more than 30 years showed a polyclonal CD8$^+$ T-cell response against HIV and CMV. This cellular antiviral immune response was strongest during periods of HIV-1 replication and remained detectable during periods of HIV-1 quiescence. We hypothesize that the consistent cellular anti-HIV-1 response in combination with highly active antiretroviral therapy ensures stability and survival of these chronically HIV-1–infected hemophilia patients.

Key words: CMV; HIV-specific CD8$^+$ T lymphocytes in blood; long-term HIV-infected hemophilia patients; stable disease

Introduction

At the end of the 1970s and early 1980s, about half of all German hemophilia patients were infected with human immunodeficiency virus (HIV) through contaminated clotting factor preparations originating from the United States. Since 1983 we have been investigating the immune system of patients treated at the Heidelberg Hemophilia Center.[1–3] During 30 years of follow-up, we observed that patients developed autoantibodies against CD4$^+$ T lymphocytes, alloantibodies against HLA, autoantibodies against IgG and Fab, CD4 helper cell defects, cytokine abnormalities, macrophage and CD4$^+$ lymphocyte dysfunctions associated with surface gp120-complement complexes, increased soluble Fas plasma levels, and apoptotic and nonapoptotic CD4$^+$ T-cell depletion; during highly active antiretroviral therapy (HAART), they had decreasing sCD30 plasma levels and increasing dendritic cells (DC) and regulatory T-cell (Treg) blood levels.[1–14] Interestingly, long-term HIV$^+$ hemophilia patients and HIV$^-$ hemophilia patients showed four and two times as many activated CD3$^+$CD8$^+$ DR$^+$ T lymphocytes in the blood, respectively, compared with healthy controls tested in parallel.[4] In a recently published manuscript,[4] we speculated that HIV$^-$ hemophilia patients might have an increased cytotoxic T-cell response because

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ABBREVIATIONS: CMV, cytomegalovirus; DC, dendritic cells; EBV, Epstein-Barr virus; FITC, fluorescein isothiocyanate; HAART, highly active antiretroviral therapy; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IFN, interferon; IL, interleukin; PBL, peripheral blood lymphocytes; PBS, phosphate-buffered saline; TGF, transforming growth factor; TNF, tumor necrosis factor; Treg, regulatory T cell.

they were stimulated by clotting factor preparations, inactivated virus particles contaminating the clotting factor preparations, and chronic virus infections acquired in the era before virus inactivation of clotting factor preparations was practicable. In HIV^+ hemophilia patients we found that interleukin (IL)-12^+ DCs were strongly associated with high $CD3^+CD8^+{}^{DR+}$ blood lymphocyte counts, whereas HIV^- hemophilia patients showed only a weak association of IL-12^+ DCs with $CD3^+CD8^+DR^+$ peripheral blood lymphocytes (PBLs). The $CD3^+CD8^+DR^+$ response was strongly related with HIV-1 viral load, and we speculated that this might represent a broad polyclonal cellular immune response, including an antiretroviral response that might contribute to the good clinical outcome of these patients.[4]

To substantiate our hypothesis, we used six HIV-specific and two cytomegalovirus (CMV)-specific pentamers in the present study to analyze the frequency of HIV- and CMV-specific $CD8^+$ T lymphocytes in the blood of HIV-infected hemophilia patients infected more than 30 years ago.

Methods

HIV^+ hemophilia patients and healthy controls

We studied 48 HIV^+ hemophilia patients during the years 2010–2012. In parallel, lymphocyte subsets were studied in 102 healthy controls and plasma cytokines in 39 healthy individuals, immune complex–loaded $CD4^+$ lymphocytes in 19, and $CD8^+$ lymphocytes reactive with HIV- and CMV-specific pentamers in 25. Patient mean age was 46 ± 8 years ($\pm$SD; range 29–70 years), that of healthy controls 40 ± 13 years (range 21–65 years). The patients were all men infected with HIV at the end of the 1970s and in the early 1980s through virus-contaminated clotting factor products and are part of an ongoing long-term study. All patients received antiretroviral treatment consisting of combinations of antiretroviral drugs. At the time of the investigation, 15 HIV^+ patients had a retroviral load below the detection limit of the test (< 10 HIV-1 RNA copies/mL), and 33 patients had a retroviral load of 19–300,000 HIV-1 RNA copies/mL. All patients gave informed consent for the tests performed within this study. The study was approved by the local ethical committee and conducted in adherence to the Declaration of Helsinki.

HLA typing

HLA determinants of patients were typed using a low resolution serological standard methods. 14 (29%) patients possessed HLA-A1, 22 (46%) A2, 17 (35%) A3, 9 (19%) A11, 3 (6%) A23, 7 (15%) A24, 3 (6%) A25, 4 (8%) A26, 1 (2%) A28, 4 (8%) A29, 1 (2%) A31, 2 (4%) A32, 2 (4%) A33, 2 (4%) A69, 11 (23%) B7, 10 (21%) B8, 5 (10%) B13, 1 (2%) B14, 4 (8%) B18, 4 (8%) B27, 12 (25%) B35, 1 (2%) B37, 2 (4%) B38, 2 (4%) B39, 1 (2%) B41, 9 (19%) B44, 1 (2%) B49, 2 (4%) B50, 5 (10%) B51, 3 (6%) B52, 1 (2%) B55, 2 (4%) B57, 1 (2%) B58, 4 (8%) B60, 1 (2%) B61, 5 (10%) B62, and 2 (4%) B65.

Pentamers

Determination of pentamer-specific $CD8^+$ blood lymphocytes was performed according to the instructions of the manufacturer. We used the following pentamers: A*02:01 SLYNTVATL (epitope origin HIV-1 gag p17 76-84), A*02:01 ILKEPVHGV (HIV-1 RT 476-484), A*03:01 QVPLRPMTYK (HIV-1 nef 73-82), B*07:02 IPRRIRQGL (HIV-1 env gp120 848-856), B*08:01 FLKEKGGL (HIV-1 nef 90-97), B*27:05 KRWIILGLNK (HIV-1 gag p24 265-274) for detection of HIV-specific cellular responses and A*0201 NLVPMVATV (HCMV pp65 495-504) and A*24:02 VYALPLKML (HCMV pp65 113-121) for the determination of CMV-reactive $CD8^+$ blood lymphocytes. First, 10 μL of PE-labeled Pro5® pentamers (ProImmune, Oxford, United Kingdom) were pipetted into 100 μL of heparinized whole blood, vortexed, and incubated at room temperature in the dark for 10 min. One microliter of fluorescein isothiocyanate (FITC)-labeled anti-CD8 monoclonal antibody (BD Biosciences, Heidelberg, Germany) was added, vortexed, and incubated at room temperature in the dark for 20 min. Two milliliters of lysis buffer (BD Pharmlyse, BD Biosciences) was added, vortexed, and incubated at room temperature in the dark for 2 min. Samples were centrifuged at 291 g for 8 min, and the supernatant was decanted. Cells were washed twice with 1.5 mL of phosphate-buffered saline (PBS)/bovine serum albumin wash buffer and centrifuged at 291 g for 8 min. Supernatant was decanted and 200 μL of PBS was added. The samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences).

Determination of plasma cytokines, soluble cytokine receptors, and soluble cytokine receptor antagonists

Plasma IL-2, IL-5, IL-6, IL-8, IL-10, transforming growth factor (TGF)-β_2, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α were determined by enzyme-linked immunosorbent assay. All cytokines were measured using Quantikine kits (R&D Systems, Wiesbaden, Germany). Plasma was snap frozen within 2 h after the blood was drawn and stored at -30°C until testing.

Determination of lymphocyte subpopulations

$CD3^+CD16^-56^-CD19^-CD45^+$, $CD3^-CD16^+56^+CD19^-CD45^+$, $CD3^-CD16^-56^-CD19^+CD45^+$, $CD3^+CD4^+CD8^-CD45^+$, and $CD3^+CD4^-CD8^+CD45^+$ lymphocyte subsets were defined in heparinized whole blood using four-color fluorescence flow cytometry and antibodies purchased from Becton Dickinson/Pharmingen (BD, Heidelberg, Germany).

Determination of Ig, IgM, IgG, C3d, and gp120 on $CD4^+$ and $CD8^+$ T lymphocytes

The proportion of immunoglobulin-positive $CD4^+$ cells in peripheral blood was determined using double fluorescence flow cytometry. First, 100 μL of whole blood was incubated with 10 μL of PE-conjugated anti-CD3, anti-CD4, or anti-CD8 (all BD Biosciences) monoclonal antibody for 30 min at 4°C. Erythrocytes were lysed by the addition of NH_4Cl solution for 15 min, the cells were washed with PBS, and 50 μL of FITC-labeled goat-anti-human-Ig (Medac, Hamburg, Germany), goat-anti-human-IgG (Tago, Burlingame, CA), goat-anti-human-IgM (Medac), rabbit-anti-human-C3d (Dakopatts, Hamburg, Germany), or 10 μL sheep-anti-gp120 (Biochrom, Berlin, Germany) was added. Sheep-anti-gp120 was used undiluted, the other antibodies were diluted 1:40. The cells were incubated for another 30 min at 4°C, washed, and analyzed by flow cytometry (FACScan, BD Biosciences). The gate setting for background staining was adjusted to < 1% $CD3^+IgG^+$ control lymphocytes, and this gate was used for all subsequent analyses. Finally, 150 healthy controls

Table 1. Demographic, Virological, and Immunological Data of HIV$^+$ Hemophilia Patients With or Without Detectable HIV-1 Viral Load

Parameter	*Patients with*		*Healthy controls* (n = *102*)	p *<10 vs. ≥10 HIV-1 RNA copies/mL plasma*	p *Healthy controls vs. <10 HIV-1 RNA copies/mL plasma*	p *Healthy controls vs. ≥10 HIV-1 RNA copies/mL plasma*
	<10 HIV-1 RNA copies/mL plasma (n = *33*)	*≥10 HIV-1 RNA copies/mL plasma* (n = *15*)				
Viral load (HIV-1 RNA copies/mL plasma)	0	84866 ± 112313 (19–300000)	—	**< 0.001**	—	—
CD45$^+$/μL	1701 ± 745 (417–3763)	1561 ± 919 (452–3395)	1924 ± 647 (980–3938)	n.s.	n.s.	0.012
CD3$^+$/μL	1208 ± 514 (154–2678)	1132 ± 747 (177–2501)	1388 ± 479 (672–2805)	n.s.	n.s.	0.023
CD4$^+$/μL	474 ± 210 (54–1089)	430 ± 381 (9–1220)	864 ± 325 (405–1779)	n.s.	**< 0.001**	**< 0.001**
CD8$^+$/μL	699 ± 388 (96–2156)	684 ± 455 (154–1506)	483 ± 257 (141–1886)	n.s.	**< 0.001**	n.s.
CD16$^+$CD56$^+$/μL	169 ± 112 (19–490)	156 ± 154 (44–611)	237 ± 150 (50–809)	n.s.	**0.009**	**0.001**
CD19$^+$/μL	295 ± 248 (33–1129)	246 ± 126 (61–475)	256 ± 119 (74–778)	n.s.	n.s.	n.s.
CD3$^+$CD25$^+$/μL	148 ± 76 (33–416)	126 ± 80 (23–306)	194 ± 83 (61–485)	n.s.	**0.001**	**< 0.001**
CD3$^+$DR$^+$/μL	201 ± 124 (67–588)	332 ± 336 (31–1331)	107 ± 76 (26–638)	n.s.	**< 0.001**	**0.001**
CD4$^+$DR$^+$/μL	42 ± 22 (8–105)	67 ± 69 (14–244)	39 ± 22 (10–148)	n.s.	n.s.	n.s.
CD8$^+$DR$^+$/μL	148 ± 106 (37–490)	266 ± 284 (16–1135)	68 ± 63 (0–551)	n.s.	**< 0.001**	**< 0.001**
CD4/CD8 ratio	0.81 ± 0.45 (0.20–2.18)	0.69 ± 0.59 (0.04–2.20)	2.0 ± 0.91 (0.31–5.17)	n.s.	**< 0.001**	**< 0.001**
% CD4$^+$IgG$^+$	3.6 ± 3.2 (0–18)	2.5 ± 2.2 (0–7)	2.6 ± 2.3 (0–7)[a]	n.s.	n.s.	n.s.
% CD4$^+$IgM$^+$	4.6 ± 4.9 (0–22)	5.5 ± 5.9 (1–19)	2.8 ± 3.4 (0–12)[a]	n.s.	**0.005**	0.021
% CD4$^+$gp120$^+$	0.70 ± 1.5 (0–7)	0.79 ± 1.3 (0–4)	1.9 ± 0.9 (1–4)[a]	n.s.	**< 0.001**	**0.004**
% A*02:01 SLYNTVATL CD8$^+$	0.26 ± 0.62 (0–3.6)	0.41 ± 0.65 (0.02–2.4)	0.04 ± 0.04 (0–0.15)[a]	n.s.	**0.006**	**< 0.001**
% A*02:01 ILKEPVHGV CD8$^+$	0.15 ± 0.17 (0.0–0.66)	0.21 ± 0.36 (0.01–1.35)	0.03 ± 0.03 (0–0.11)[a]	n.s.	**0.003**	**< 0.001**
% A*03:01 QVPLRPMTYK CD8$^+$	0.43 ± 0.96 (0.0–5.0)	0.30 ± 0.39 (0.0–1.48)	0.11 ± 0.10 (0–0.39)[a]	n.s.	n.s.	0.022
% B*07:02 IPRRIRQGL CD8$^+$	0.13 ± 0.16 (0–0.59)	0.11 ± 0.14 (0–0.56)	0.02 ± 0.02 (0–0.08)[a]	n.s.	**< 0.001**	**< 0.001**
% B*08:01 FLKEKGGL CD8$^+$	0.16 ± 0.21 (0–0.91)	0.39 ± 1.2 (0.02–4.6)	0.02 ± 0.03 (0–0.12)[a]	n.s.	**< 0.001**	**< 0.001**
% B*27:05 KRWIILGLNK CD8$^+$	0.20 ± 0.30 (0–1.6)	0.22 ± 0.26 (0–0.91)	0.04 ± 0.04 (0–0.15)[a]	n.s.	**0.001**	**< 0.001**
% A*02:01 NLVPMVATV CD8$^+$ (CMV)	0.37 ± 0.69 (0–3.7)	0.41 ± 0.65 (0.02–2.4)	0.07 ± 0.08 (0–0.33)[a]	n.s.	0.010	**0.001**
% A*24:02 VYALPLKML CD8$^+$ (CMV)	0.15 ± 0.15 (0–0.60)	0.22 ± 0.22 (0.0–0.60)	0.06 ± 0.06 (0–0.20)[a]	n.s.	n.s.	0.021

All data are given as mean ± SD with range in parentheses. Data were analyzed using Mann-Whitney U test. *p*-Values < 0.01 are presented in boldface.

[a]Frequency of pentamer-reactive CD8$^+$ blood lymphocytes was determined in 25 healthy control individuals; IgG, IgM, and gp120 load of CD4$^+$ blood lymphocytes were determined in 19 healthy controls.

HIV, human immunodeficiency virus; CMV, cytomegalovirus; n.s., not significant.

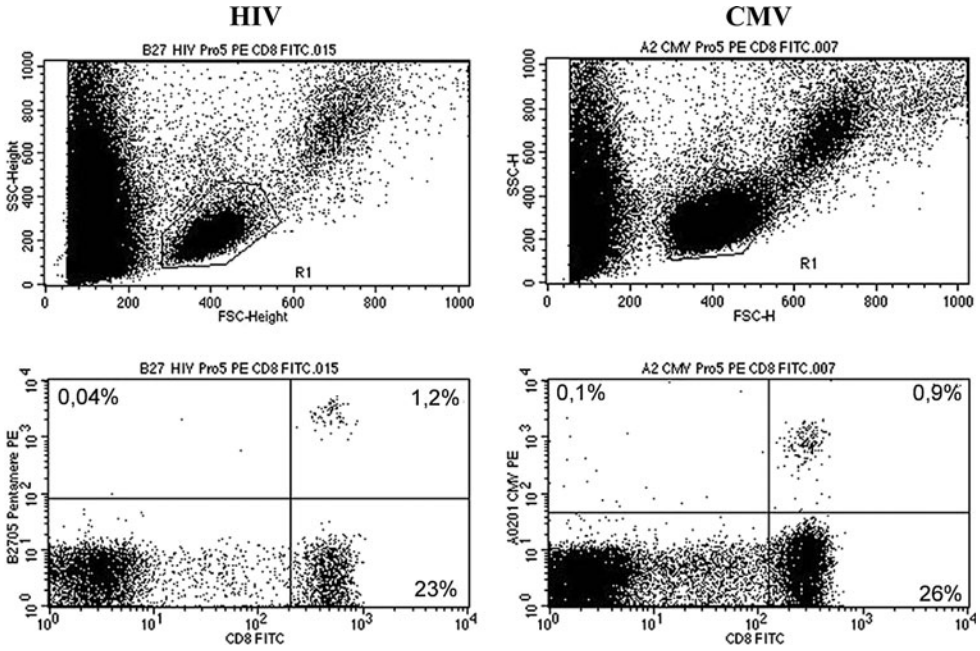

FIG. 1. Flow cytometric determination of HIV and cytomegalovirus (CMV) pentamer–specific $CD8^+$ blood lymphocytes. Peripheral blood lymphocytes (PBLs) in heparinized whole blood samples were gated using an FSC/SSC dot plot and analyzed further on for their reactivity with HIV and CMV pentamers as well as CD8 monoclonal antibody. More than 0.3% double positive HIV^+CD8^+ or CMV^+ $CD8^+$ blood lymphocytes were considered as positive test results.

whose blood samples were tested in a previous series of experiments showed a median (interquartile range) of $CD3^+$ IgG^+ 1.0% (0%), $CD4^+IgM^+$ 1.0% (1%), $CD4^+IgG^+$ 1.0% (1%), $CD4^+C3d^+$ 5.0% (5%), $CD4^+gp120^+$ 1.0% (1%), $CD8^+IgM^+$ 2.0% (3%), $CD8^+IgG^+$ 2.0% (4%), and $CD8^+$ $C3d^+$ 15.0% (10%) lymphocytes in the blood. Tests resulting in >30% double-stained cells were defined as autoantibody or immune complex positive.

Determination of HIV-1 RNA copies in plasma

HIV-1 RNA was measured using the NucliSens EasyQ HIV-1 V2.0 kit (bioMérieux, Nürtingen, Germany). According to the manufacturer's instructions, HIV-1 RNA was quantified using a 1-mL plasma sample. Sensitivity of the assay is <10 copies using samples of 1 mL of plasma.

Statistical analysis

Wilcoxon rank sum test, Spearman rank correlation test, Fisher's exact test for 3×2 contingency table and chi-square test were used for statistical analysis applying the Statistical Program for the Social Sciences (SPSS, Chicago, IL). Adjustment for multiple testing was done according to the method of Bonferroni. *P*-values of <0.01 were considered significant and are bolded in the tables.

Results

Demographical, virological, and immunological data of hemophilia patients infected with HIV 30 years ago

HIV^+ hemophilia patients were grouped according to whether they had detectable (≥10 HIV-1 RNA copies/mL serum, $n=15$) or undetectable retroviral load (<10 HIV-1 RNA copies/ml serum, $n=33$; Table 1). Pentamer tests were performed multiple times during the last 2 years; the latest investigation of each patient was used for the current analysis. Flow cytometric determination of pentamer-specific $CD8^+$ blood lymphocytes is depicted in Fig. 1. Compared with healthy controls, both groups of HIV^+ patients had lower absolute counts of $CD4^+$, $CD16^+CD56^+$, and $CD3^+CD25^+$ lymphocytes and higher absolute counts of $CD3^+DR^+$ and $CD8^+DR^+$ lymphocytes (all $p<0.010$; Table 1, Mann–Whitney *U* test), as well as higher proportions of $CD8^+$ blood lymphocytes reactive with HIV or CMV pentamers ($p<0.010$ for the pentamers A*02:01 SLYNTVATL, A*02:01 ILKEPVHGV, B*07:02 IPRRIRQGL, B*08:01 FLKEKGGL, B*27:05 KRWIILGLNK, A*02:01 NLVPMVATV [CMV]; Table 1). $CD4^+$ blood lymphocytes with abnormally high IgG/gp120 immune-complex load were no longer detectable in the blood of the two patient groups (versus healthy controls: $p=$ not significant [n.s.]), confirming the stable clinical course of these long-term HIV-infected patients (Table 1). When the two patient groups were compared with each other, there was no difference in absolute counts of $CD45^+$, $CD3^+$, $CD3^+$ DR^+, $CD3^+CD25^+$, $CD4^+DR^+$, $CD8^+$, and $CD8^+DR^+$ T lymphocytes; $CD19^+$ B lymphocytes and $CD16^+CD56^+$ NK cell subsets; $CD8^+$ lymphocytes reactive with HIV or CMV pentamers; or proportions of $CD4^+$ blood lymphocytes coated with IgG, IgM, and/or gp120 ($p=$n.s., Table 1). The data suggest that long-term HIV^+ hemophilia patients with undetectable HIV-1 viral load are similar with respect to these immune parameters as long-term HIV^+ hemophilia patients with detectable HIV-1 viral load. However, both groups of HIV^+ patients have abnormally high proportions of circulating activated $CD8^+DR^+$ lymphocytes as well as circulating $CD8^+$ lymphocytes reactive with HIV-1 and CMV.

Association of HIV-specific $CD8^+$ T lymphocytes with other immune parameters

High $CD8^+DR^+$ blood lymphocytes were associated with high CMV-specific $CD8^+$ lymphocytes (A*02:01 NLVPMVATV $p=0.003$; A*24:02 VYALPLKML $p=0.020$; Table 2) and tended to be associated with HIV-specific $CD8^+$ lymphocytes (B*07:02 IPRRIRQGL, $p=0.046$; B*08:01

Table 2. Association of HIV-Specific $CD8^+$ Peripheral Blood Lymphocytes with Virological and Immunological Parameters in 48 HIV^+ Hemophilia Patients

	*% A*02:01 SLYNTVATL $CD8^+$* r (p)	*% A*02:01 ILKEPVHGV $CD8^+$* r (p)	*% A*03:01 QVPLRPMTYK $CD8^+$* r (p)	*% B*07:02 IPRRIRQGL $CD8^+$* r (p)	*% B*08:01 FLKEKGGL $CD8^+$* r (p)	*% B*27:05 KRWIILGLNK $CD8^+$* r (p)	*% A*02:01 NLVPMVATV $CD8^+$ (CMV)* r (p)	*% A*24:02 VYALPLKML $CD8^+$ (CMV)* r (p)
$CD45^+/\mu L$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
$CD3^+/\mu L$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.313 (0.032)	n.s.
$CD3^+DR^+/\mu L$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.358 (0.014)	n.s.
$CD3^+25^+/\mu L$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
$CD4^+/\mu L$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
$CD4^+DR^+/\mu L$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.318 (0.030)	n.s.
$CD8^+/\mu L$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.473 (**0.001**)	n.s.
$CD8^+DR^+/\mu L$	n.s.	n.s.	n.s.	0.302 (0.046)	0.353 (0.014)	0.297 (0.040)	0.429 (**0.003**)	0.361 (0.020)
$CD16^+56^+/\mu L$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
$CD19^+/\mu L$	n.s.	n.s.	−0.309 (0.034)	−0.397 (**0.008**)	−0.472 (**0.001**)	−0.472 (**0.001**)	n.s.	n.s.
$CD4^+/CD8^+$	−0.304 (0.35)	n.s.	−0.375 (**0.009**)	−0.315 (0.037)	−0.298 (0.040)	n.s.	n.s.	n.s.
Viral load (HIV-1 RNA copies/mL plasma)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
% A*02:01 SLYNTVATL $CD8^+$	—	0.682 (<**0.001**)	0.619 (<**0.001**)	0.469 (**0.001**)	0.520 (<**0.001**)	0.373 (**0.009**)	0.3084 (0.035)	n.s.
% A*02:01 ILKEPVHGV $CD8^+$	0.682 (<**0.001**)	—	0.551 (<**0.001**)	0.421 (**0.005**)	0.432 (**0.002**)	n.s.	0.308 (0.037)	0.419 (**0.006**)
% A*03:01 QVPLRPMTYK $CD8^+$	0.619 (<**0.001**)	0.551 (<**0.001**)	—	0.442 (**0.003**)	0.581 (<**0.001**)	0.515 (<**0.001**)	0.363 (0.013)	0.431 (**0.005**)
% B*07:02 IPRRIRQGL $CD8^+$	0.469 (**0.001**)	0.421 (**0.005**)	0.442 (**0.003**)	—	0.686 (<**0.001**)	0.633 (<**0.001**)	0.371 (0.014)	0.536 (**0.001**)
% B*08:01 FLKEKGGL $CD8^+$	0.520 (<**0.001**)	0.432 (**0.002**)	0.581 (<**0.001**)	0.686 (<**0.001**)	—	0.699 (<**0.001**)	0.481 (**0.001**)	0.317 (0.043)
% B*27:05 KRWIILGLNK $CD8^+$	0.373 (**0.009**)	n.s.	0.515 (<**0.001**)	0.633 (<**0.001**)	0.699 (<**0.001**)	—	0.426 (**0.003**)	0.501 (**0.001**)
% A*02:01 NLVPMVATV $CD8^+$ (CMV)	0.308 (0.035)	0.308 (0.037)	0.363 (0.013)	0.371 (0.014)	0.481 (**0.001**)	0.426 (**0.003**)	—	0.359 (0.023)
% A*24:02 VYALPLKML $CD8^+$ (CMV)	n.s.	0.419 (**0.006**)	0.431 (**0.005**)	0.536 (**0.001**)	0.317 (0.043)	0.501 (**0.001**)	0.359 (0.023)	—

Data were analyzed using Spearman rank correlation test. *p*-Values <0.01 are presented in boldface.

Table 3. Categorization of HIV-Infected Hemophilia Patients With or Without HIV- and Cytomegalovirus-Reactive CD8$^+$ Peripheral Blood Lymphocytes and With or Without HIV-1 Viral Load

48 HIV-infected hemophilia patients							
23 (48%) patients without HIV-reactive CD8$^+$ PBLs		25 (52%) patients with HIV-reactive CD8$^+$ PBLs		27 (56%) patients without CMV-reactive CD8$^+$ PBLs		21 (44%) patients with CMV-reactive CD8$^+$ PBLs	
15 (65%) patients without HIV VL	8 (35%) patients with HIV VL	18 (72%) patients without HIV VL	7 (28%) patients with HIV VL	19 (70%) patients without HIV VL	8 (35%) patients with HIV VL	14 (67%) patients without HIV VL	7 (33%) patients with HIV VL

VL, viral load.

FLKEKGGL, $p = 0.014$; B*27:05 KRWIILGLNK, $p = 0.040$; Table 2). Interestingly, high HIV pentamer–specific CD8$^+$ lymphocytes were strongly associated with low CD19$^+$ B lymphocytes counts in the blood (B*27:05 KRWIILGLNK, $p = 0.001$; B*08:01 FLKEKGGL, $p = 0.001$; B*07:02 IPRRIRQGL, $p = 0.008$; A*03:01 QVPLRPMTYK, $p = 0.034$; Table 2). HIV and CMV pentamer–specific CD8$^+$ lymphocytes frequencies were strongly associated with each other (Table 2). Our findings suggest that activated CD8$^+$DR$^+$ blood lymphocytes are directed against CMV as well as HIV, and that patients with pentamer-reactive CD8$^+$ blood lymphocytes have polyclonal responses against different epitopes of these viruses. Cellular anti-HIV and anti-CMV responses are strongest in patients with low blood B-lymphocyte counts.

Proportion of patients with HIV-specific CD8$^+$ T lymphocytes in the blood

Based on measurements in healthy controls (Table 1) and according to manufacturer instructions, a cutoff of >0.3% (of total PBLs) pentamer-reactive CD8$^+$ blood lymphocytes was defined as a positive test result (Fig. 1). Using this definition, 23 of 48 (48%) patients showed no CD8$^+$ response to HIV-specific peptides, seven (15%) reacted with one HIV pentamer, nine (19%) with two, six (13%) with three, two (4%) with four, and one (2%) with five HIV pentamers. With respect to CMV, 27 (56%) patients had no detectable CMV-specific CD8$^+$ lymphocytes, 15 (31%) reacted with one and six (13%) with two CMV pentamers.

Of the 23 patients with undetectable HIV pentamer–specific CD8$^+$ lymphocytes, 15 (65%) had undetectable HIV-1 viral load (Table 3). Of the 27 patients with undetectable CMV pentamer–specific CD8$^+$ blood lymphocytes, 19 (70%) showed undetectable HIV-1. These data indicate that approximately half of the long-term HIV-infected hemophilia patients had no detectable HIV- or CMV-reactive CD8$^+$ lymphocytes in the blood, two thirds thereof during HIV-1 quiescence with no detectable viral load. When, in addition, patients with a positive cellular anti-HIV response were analyzed, 18 of 25 (72%) patients with detectable HIV-specific CD8$^+$ lymphocytes and 14 of 21 (67%) patients with detectable CMV-specific CD8$^+$ lymphocytes had no detectable HIV-1 replication (Table 3). Thus, strong antiviral CD8$^+$ lymphocyte responses occurred in patients with detectable as well as undetectable HIV-1 load.

Table 4. CD4$^+$ and CD8$^+$DR$^+$ Lymphocyte Counts in Eight Patients with Detectable Viral Load But No HIV Pentamer–Reactive CD8$^+$ Blood Lymphocytes

Patient	*% HIV pentamer-reactive CD8$^+$ PBLs with respect to all PBLs*	*HIV-1 copies/mL*	*CD4$^+$/μL*	*CD8$^+$ DR$^+$/μL*
1	≤0.3	140	515	213
2	≤0.3	28	1220	366
3	≤0.3	260	1154	136
4	≤0.3	54	500	34
5	≤0.3	5000	20	192
6	≤0.3	120,000	371	1135
7	≤0.3	470	287	318
8	≤0.3	24	685	98

Among the eight patients with detectable HIV-1 load but no HIV-reactive CD8$^+$ lymphocytes in the blood, there was only one conspicuous patient with very low CD4$^+$ lymphocytes (20/μL), high HIV-1 load (5000 copies/mL), and, interestingly, high CD8$^+$DR$^+$ lymphocyes (192/μL; Table 4). The other seven patients showed >250/μL CD4$^+$ lymphocytes, six of them <500 HIV-1 copies/mL, and four >193/μL CD8$^+_{DR+}$ lymphocytes, suggesting reasonable disease stability. Table 5 shows the profile of patient 1 from Table 4. The patient showed a simultaneous loss of HIV pentamer–reactive CD8$^+$ lymphocytes and an increase in HIV-1 viral load, suggesting that the HIV pentamer–reactive CD8$^+$ lymphocytes may have protected the patient from increased retroviral replication.

Analysis of multiple consecutive investigations in HIV$^+$ hemophilia patients

The 48 HIV$^+$ patients were investigated a total of 212 times (1–11 investigations per patient, Fig. 2) during a period of 31 months. In this rather small series of irregular investigations, 22 (46%) patients showed consistently undetectable HIV-1 viral load, eight (17%) patients showed consistently detectable HIV-1 viral load, and 18 (38%) patients alternated between periods of detectable and undetectable retroviral load (Fig. 2).

Patients with consistently undetectable HIV-1 viral load tended to show lower frequencies of polyclonal anti-HIV responses than patients experiencing periods of retroviral replication. When patients with polyclonal anti-HIV responses were defined as showing ≥50% positive test results based on all tests with HIV-1 pentamers during the 31-month observation period, only 1 of 22 (4.5%) patients with consistently undetectable HIV-1 viral load showed a strong and broad

TABLE 5. SIMULTANEOUS INCREASE OF HIV-1 LOAD AND DECREASE OF PENTAMER-REACTIVE CD8$^+$ LYMPHOCYTES IN AN HIV$^+$ HEMOPHILIA PATIENT

Date	HIV-1 copies/mL	CD4$^+$/μL	CD8$^+$ DR$^+$/μL	% A*02:01 SLYNTVATL CD8$^+$	% A*02:01 ILKEPVHGV CD8$^+$	% A*03:01 QVPLRPMTYK CD8$^+$	% B*07:02 IPRRIRQGL CD8$^+$	% B*08:01 FLKEKGGL CD8$^+$	% B*27:05 KRWIILGLNK CD8$^+$	% A*02:01 NLVPMVATV CD8$^+$ (CMV)	% A*24:02 VYALPLKML CD8$^+$ (CMV)
July 29, 2010	0	823	235	0.65	0.61	0.40	—	0.41	0.66	1.87	0.51
October 4, 2010	0	650	210	0.72	0.33	0.36	0.18	0.08	0.08	0.28	0.08
April 11, 2011	0	502	208	0.50	0.35	0.53	0.39	0.28	0.27	0.84	0.17
August 19, 2011	0	509	175	0.85	0.64	0.36	0.00	0.02	0.02	0.56	0.20
April 4, 2012	140	515	213	0.19	0.03	0.08	0.13	0.04	0.16	0.17	0.06

CD8$^+$ lymphocyte response against HIV pentamers, whereas 3 of 18 (17%) patients with alternating periods of detectable and undetectable retroviral replication and three of eight (38%) patients with consistently detectable HIV-1 viral load exhibited polyclonal anti-HIV responses ($p = 0.077$, Fisher's exact test for 2×3 contingency table). The corresponding results with CMV pentamers were 5 of 22 (23%), 5 of 18 (28%), and 2 of 8 (25%) patients, suggesting that HIV-1 replication induces anti-HIV but not anti-CMV CD8$^+$ lymphocyte responses. Although our results did not reach statistical significance, they suggest that patients with consistent retroviral quiescence lack broad polyclonal cellular responses against HIV, whereas patients with retroviral replication tend to form CD8$^+$ lymphocytes against several different HIV pentamers.

Plasma cytokines and HIV-specific CD8$^+$ T lymphocytes

Because high HIV and CMV pentamer–reactive CD8$^+$ blood lymphocytes were associated with high activated CD8$^+$DR$^+$ T lymphocyte and low CD19$^+$ B lymphocyte counts, we studied plasma cytokine levels in 42 patients to investigate whether there was a relationship with a pronounced Th1 cytokine pattern (Table 6). Patients with ($n = 11$) or without ($n = 31$) detectable HIV-1 load had similar IL-2, IL-5, IL-6, IL-8, IL-10, IFN-γ, TGF-β1, and TNF-α plasma levels (p = n.s.). IL-6 and TNF-α plasma levels were lower in healthy controls ($n = 39$; IL-6, 1.1 ± 1.6 pg/mL; TNF-α, 0.85 ± 0.96 pg/mL) than in patients with (IL-6, 3.6 ± 5.2 pg/mL, $p = 0.001$; TNF-α, 3.4 ± 7.2 pg/mL, $p = 0.022$) or without HIV-1 viral load (IL-6, 2.7 ± 2.6 pg/mL, $p < 0.001$; TNF-α, 2.1 ± 4.7 pg/mL, $p = 0.020$). IL-8 was normal in patients without HIV-1 load and lower in patients with HIV-1 load compared to healthy individuals (4.5 ± 10 pg/mL vs. 13 ± 31 pg/mL, $p = 0.042$). Plasma levels of the other cytokines were similar in both patient groups and controls (p = n.s.). The data show evidence for strong monocyte and macrophage activation but normal Th1 and Th2 cytokine levels in these HIV$^+$ patients after ≥ 30 years of follow up.

High monocyte- and macrophage-derived plasma cytokine levels were associated with HIV pentamer–reactive CD8$^+$ lymphocytes only in patients with HIV-1 load but not in patients without HIV-1 load (Table 6). TNF-α was associated with A*03:01 QVPLRPMTYK ($p = 0.025$), B*08:01 FLKEKGGL ($p = 0.024$), and B*27:05 KRWIILGLNK ($p = 0.004$), IL-6 with B*07:02 IPRRIRQGL ($p = 0.013$), and TGF-β1 with B*27:05 KRWIILGLNK ($p = 0.042$), indicating that CD8$^+$ lymphocyte responses occurred during periods of normal Th1 and Th2 plasma cytokine levels and increased monocyte and macrophage activation.

*Reactions with A*02:01 pentamers in HLA-A2$^+$ and HLA-A2$^-$ patients*

The highest sensitivity of pentamer testing should be achieved when pentamer and patient lymphocytes are HLA identical. We investigated the number of positive reactions (>0.3% pentamer-reactive CD8$^+$ lymphocytes of all PBLs) with two different HLA-A*02:01 HIV pentamers in 22 HLA-A2$^+$ and 26 HLA-A2$^-$ patients. Of the 22 HLA-A2$^+$ patients, 7 (32%) without and 3 (14%) with HIV-1 load showed positive reactions, indicating that a fraction of HLA-A2$^+$ patients with as well as without HIV-1 load have circulating CD8$^+$

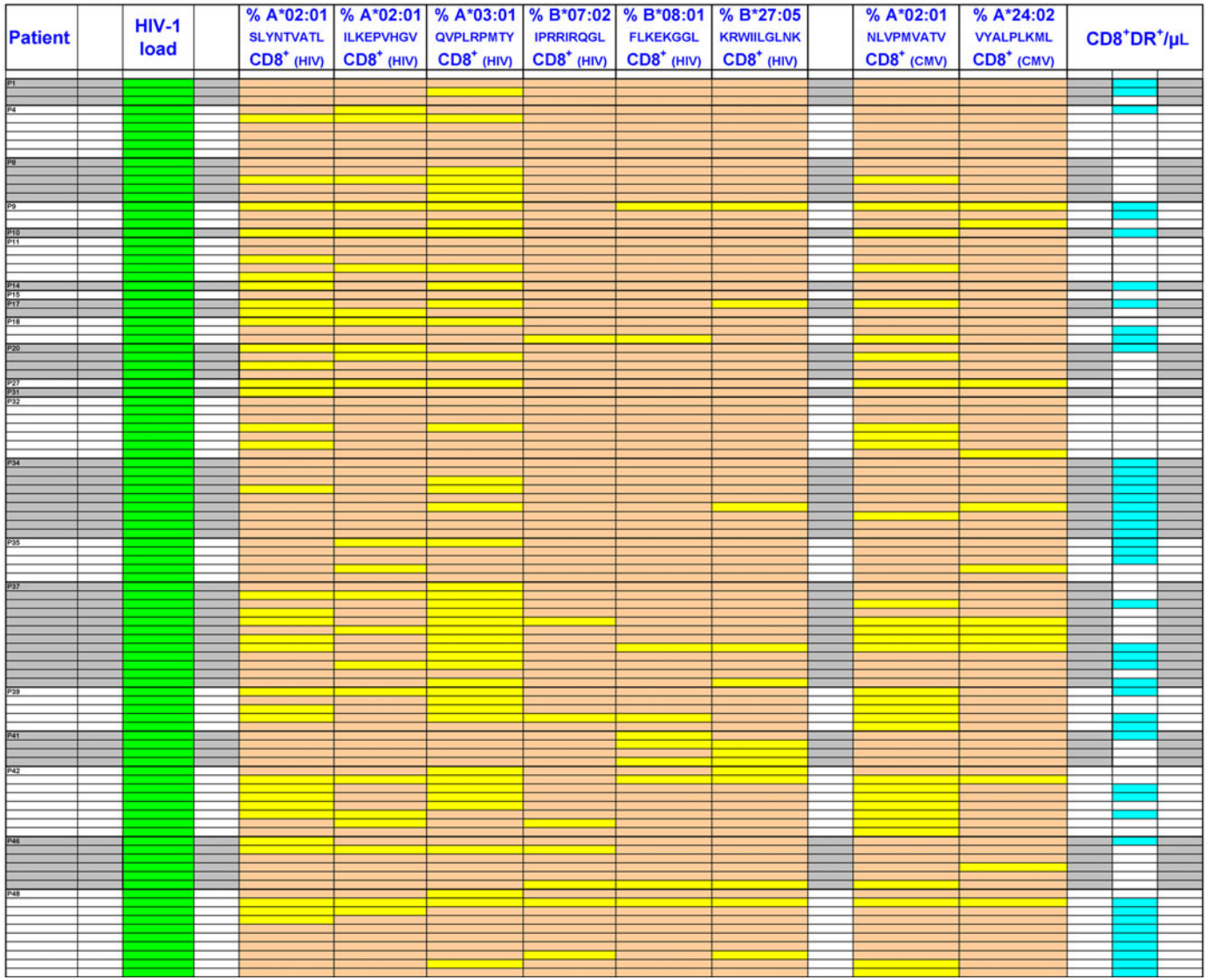

FIG. 2. Multiple investigations of HIV$^+$ hemophilia patients. During a period of 31 months, 48 long-term HIV-infected hemophilia patients were studied 212 times in total (range 1–11 investigations per patient). Each patient is depicted by a gray or, alternatively, white array of lines, and each line represents a certain investigation date. In the column entitled HIV-1 load, investigations with detectable HIV-1 viral load are marked by red background, while test results showing undetectable HIV-1 viral load are depicted by green background. In the columns representing data of pentamer staining, test results with $>0.3\%$ pentamer-specific CD8$^+$ PBLs are depicted in yellow, while test results with $\leq 0.3\%$ pentamer-specific CD8$^+$ PBLs are shown in orange. Increased absolute numbers of $>193/\mu$L CD8$^+$DR$^+$ blood lymphocytes (mean $\pm$ 2 SD of 102 healthy controls) are depicted in blue, while CD8$^+$DR$^+$ blood lymphocytes counts of $\leq 193/\mu$L in white. (*continued*)

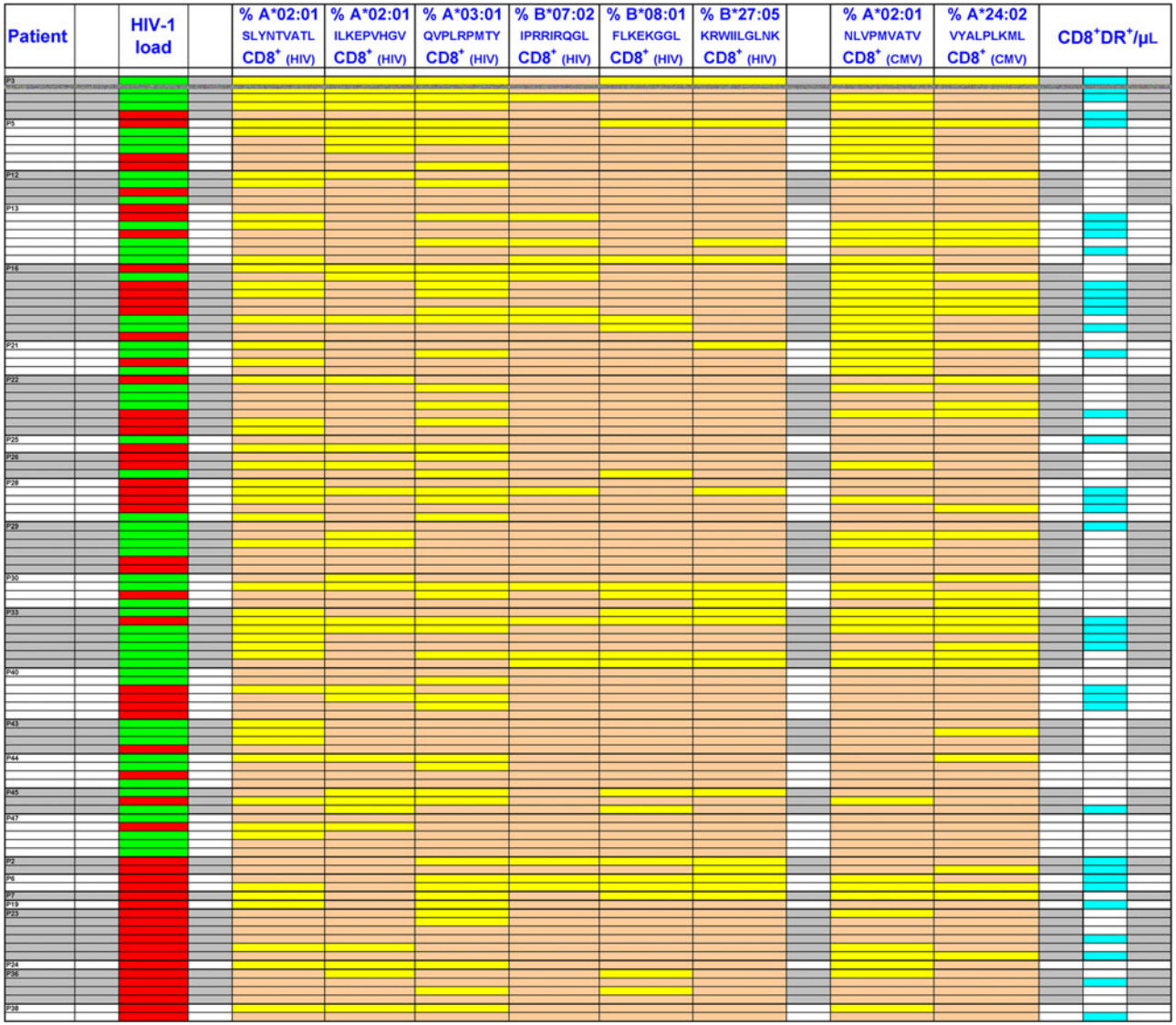

FIG. 2. (Continued).

Table 6. Association of HIV-Specific $CD8^+$ Peripheral Blood Lymphocytes with Plasma Cytokine Levels in 42 HIV^+ Hemophilia Patients

Parameter	*IL-2* r (p)	*IL-5* r (p)	*IL-6* r (p)	*IL-8* r (p)	*IL-10* r (p)	*IFN-γ* r (p)	*TGF-β1* r (p)	*TNF-α* r (p)
Patients with <10 HIV-1 copies/mL ($n=31$)								
% A*02:01 SLYNTVATL $CD8^+$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
% A*02:01 ILKEPVHGV $CD8^+$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
% A*03:01 QVPLRPMTYK $CD8^+$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
% B*07:02 IPRRIRQGL $CD8^+$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
% B*08:01 FLKEKGGL $CD8^+$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
% B*27:05 KRWIILGLNK $CD8^+$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
% A*02:01 NLVPMVATV $CD8^+$ (CMV)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
% A*24:02 VYALPLKML $CD8^+$ (CMV)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Patients with ≥10 HIV-1 copies/mL ($n=11$)								
% A*02:01 SLYNTVATL $CD8^+$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
% A*02:01 ILKEPVHGV $CD8^+$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
% A*03:01 QVPLRPMTYK $CD8^+$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.667 (0.025)
% B*07:02 IPRRIRQGL $CD8^+$	n.s.	n.s.	0.714 (0.013)	n.s.	n.s.	n.s.	n.s.	n.s.
% B*08:01 FLKEKGGL $CD8^+$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.670 (0.024)
% B*27:05 KRWIILGLNK $CD8^+$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.620 (0.042)	0.785 (**0.004**)
% A*02:01 NLVPMVATV $CD8^+$ (CMV)	n.s.	n.s.	−0.621 (0.042)	n.s.	n.s.	n.s.	n.s.	n.s.
% A*24:02 VYALPLKML $CD8^+$ (CMV)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Data were analyzed using Spearman rank correlation test. p-Values of <0.01 are presented in boldface.

lymphocytes that can react with HLA-A*02:01 HIV pentamers (Table 7). Of the 26 $HLA\text{-}A2^-$ patients, 1 (4%) patient without and 3 (12%) patients with retroviral load had $CD8^+$ lymphocytes in the blood that were reactive with HLA-A*02:01 HIV pentamers, confirming that those pentamers were also reactive with $CD8^+$ lymphocytes of some $HLA\text{-}A2^-$ patients.

Discussion

In 1996 we summarized the immunological dysfunctions of hemophilia patients who had lived with their HIV infection for more than 10 years.[15] We described a patient who lived with HIV for 16 years and who during the last 6 years had lost his $CD4^+$ and $CD8^+$ lymphocytes. $CD4^+$ lymphocytes decreased from a normal 561/μL to a strongly abnormal 27/μL, whereas $CD8^+$ lymphocytes decreased in parallel from a strongly elevated 1283/μL to a normal 306/μL. We interpreted these results as an indication that the patient had lost his cellular antiviral response, which resulted in increased viral replication and increased destruction of $CD4^+$ lymphocytes that were no longer capable of providing $CD8^+$ lymphocytes with the cytokines necessary for activation and proliferation of virus-reactive $CD8^+$ effector lymphocytes. During the following year, the patient was converted to HAART. Now, 16 years later, at the end of the year 2012, this patient showed 315/μL $CD4^+$, 834/μL $CD8^+$, and 299/μL activated $CD8^+DR^+$ lymphocytes in the absence of detectable HIV-1 viral load in the blood, indicating a recovery of the immune system during successful antiretroviral therapy. We hypothesize that the strong increase of activated $CD8^+DR^+$ lymphocytes observed in this patient is an indication of the presence of HIV-reactive $CD8^+$ lymphoctes that might be responsible for the consistently low HIV-1 viral load and the immunological and clinical stability of this (and other) hemophilia patients now living with HIV since they were infected 30 years ago.

Table 7. Number of $HLA\text{-}A2^+$ and $HLA\text{-}A2^-$ HIV^+ Hemophilia Patients Showing Positive Reactions with Pentamers A*02:01 SLYNTVATL and/or A*02:01 ILKEPVHGV

Patients	*HLA-A2⁺* (n=22)			*HLA-A2⁻* (n=26)		
No. of positive reactions with A*02:01 SLYNTVATL and/or A*02:01 ILKEPVHGV pentamers	0	1	2	0	1	2
No. of patients with <10 HIV-1 copies/mL	7	5	2	18	0	1
No. of patients with ≥10 HIV-1 copies/mL	5	2	1	4	2	1

To substantiate this hypothesis, we studied frequencies of circulating $CD8^+$ blood lymphocytes that were reactive with HIV and CMV peptides. Our data show that long-term HIV-infected patients have abnormally high frequencies of $CD8^+$ blood lymphocytes that are reactive with HIV and CMV (Table 1), forming a broad antiviral response against several HIV and CMV pentamers (Table 2). This antiviral response is mediated by $CD8^+DR^+$ lymphocyte in the blood of the patients (Table 2). Interestingly, $CD8^+$ lymphocytes reactive with HIV were not associated with the HIV-1 load in the blood: two thirds of patients with as well as two thirds of the patients lacking a cellular anti-HIV response showed quiescence of retroviral replication (Table 3). However, patient profiles based on irregular investigations showed a trend that polyclonality of the cellular anti-HIV response was associated with retroviral load, because strong and broad $CD8^+$ lymphocyte responses against HIV pentamers were more frequently observed in patients with consistently detectable HIV-1 load. Strong HIV replication seems to induce a boosted polyclonal $CD8^+$ lymphocyte response against several epitopes of HIV that probably decreases HIV replication; the response becomes oligoclonal and low level during periods of

retroviral quiescence. Interestingly, patients with HIV-1 load in the plasma as well as patients without had normal Th1 and Th2 cytokine responses. Thus anti-HIV CD8$^+$ lymphocyte responses were not associated with Th1 and Th2 cytokine levels in either patient group, indicating stability of the immune system of these patients.

To our knowledge, this is the first study describing anti-HIV CD8$^+$ T cell responses in patients living with HIV for 30 years. CD8$^+$ lymphocyte responses to HIV-specific pentamers or tetramers have been described by others, and the clinical relevance of the responses was controversial.[16–36] Studies by Gray et al.[37,38] provided evidence that persistently replicating viral populations are probably required for maintaining high frequencies of HIV-1 epitope-specific CD8$^+$ T cells in asymptomatic chronically infected individuals and that suppression of HIV-1 replication resulted in diminished frequencies of peripherally activated antigen-specific CD8$^+$ cells. Spiegel et al.[39] reported that HIV-1- and CMV-specific cytotoxic T lymphocytes can persist at high frequency for prolonged periods in the absence of circulating peripheral CD4$^+$ T cells. Kostense et al.[40] showed that most HIV-infected patients have sustained HIV-specific T-cell expansion, but that many of these cells are not functional, leaving the patient with high numbers of nonfunctional virus-specific CD8$^+$ T cells in the face of a high viral burden. Decrion et al.[41] described CD8$^+$CD28^{intermediate} T cells in the blood of chronically HIV-infected subjects that are specific for HIV, CMV, and Epstein-Barr virus (EBV) as measured by human leucocyte antigen pentamer binding and production of intracellular IFN-γ and TNF-α in response to their cognate viral peptides. Decrion et al.[41] concluded that a subset of functional effector-memory CD8$^+$ T cells specific for HIV, CMV, and EBV antigens may contribute to an efficient immune response in HIV-infected subjects. Kloverpris et al.[42] induced novel CD8$^+$ T cell responses during chronic untreated HIV-1 infection by immunization with subdominant cytotoxic T lymphocyte epitopes. Low frequencies of hepatitis C virus (HCV) pentamer-positive HCV-specific CD8$^+$ cells (0.01%–0.05%) were detected in 9 of 12 HCV-infected HIV$^+$ patients and represented mainly effector-memory PD-1-negative T cells.[43] Cross-reactive CD8$^+$ T cell recognition of HLA-A2–restricted HIV-Gag (SLYNTVATL) and HCV-NS5b (ALYDVVSKL) epitopes in individuals infected with HIV and HCV was reported by Vali et al.[44]; however, they found that the induction of cross-reactive T cells was not associated with control of disease by the heterologous virus. SenGupta et al.[45] showed that strong human endogenous retrovirus-specific T-cell responses were associated with control of HIV-1 in chronic infection. Harcourt et al.[46] identified two distinct patterns of response against HIV and HCV in a cohort of co-infected hemophiliacs: some patients had a limited response to either virus, while others generated responses to a range of HIV epitopes. HCV responses were detected only in those patients who formed multiple responses to HIV epitopes. Lack of functional or tetramer-positive HIV-specific T cells was associated with a decline in absolute CD4$^+$ T cell counts. Villacres et al.[47] described vigorous T-helper responses to CMV antigens in healthy CMV-seropositive donors but low responses in a cohort of HIV-infected patients. Potent CD8$^+$ cytotoxic T lymphocyte responses to CMV in HIV-infected patients receiving HAART were the converse of what was found in healthy CMV-seropositive subjects and thought to be the predominant adaptive immune response against CMV in HIV-infected patients.[47] With respect to HIV-specific CD4$^+$ T cells, Vingert et al.[48] described that individuals suppressing and controlling HIV for more than 10 years maintained a population of highly efficient CD4$^+$ Th1 effectors directed against Gag in spite of persistently low antigenemia, while long-term treated patients showed a loss of CD4 effector functions such as cytokine production. Iyasere et al.[49] showed that diminished proliferation of HIV-specific CD4$^+$ T cells is associated with diminished IL-2 production and is recovered by exogenous IL-2.

Taken together, this literature survey shows that the clinical relevance of HIV-reactive CD8$^+$ blood lymphocytes is controversial. However, a common thread is that anti-HIV CD8$^+$ cell responses are paralleled by anti-CMV, anti-EBV, and anti-HCV CD8$^+$ cell responses indicating partial immune competence in these patients. Our data show that even patients with detectable HIV-1 replication and $\leq 0.3\%$ HIV pentamer-reactive CD8$^+$ PBLs in the blood can remain clinically stable (Table 4). However, as shown in Table 5, these patients seem to have lost their protective CD8$^+$ lymphocyte response against HIV, as suggested by increased HIV-1 replication. The increase of CD8$^+$DR$^+$ lymphocytes in these patients might be due to microbial co-infections and reactivations of other viruses which can initiate immune activation and promote HIV replication as reported by Blanchard et al.[50]

Our approach to compare HIV pentamer–reactive CD8$^+$ lymphocyte responses of patients with consistently undetectable HIV-1 load with those of patients during periods of retroviral activity showed evidence for increased intensity as well as increased polyclonality of cellular anti-HIV responses during HIV-1 replication. We interpret the strong and polyclonal cellular anti-HIV responses as an attempt of the immune system, in the presence of HIV-1 antigen, to eliminate HIV-infected cells. After successful elimination of HIV-infected cells, the antiviral response decreased from polyclonality to oligoclonality. The fact that, 30 years after infection with HIV, 33 of 48 patients showed undetectable HIV-1 load (Table 1) might be interpreted as efficient control of HIV-1 replication by HIV-reactive CD8$^+$ cytotoxic T lymphocytes combined with HAART. Those 15 patients with detectable viral load during HAART showed polyclonal antiviral responses, probably an indicator of active antiretroviral immune responses.

One also has to consider that partial HLA incompatibility of pentamers with patient lymphocytes or low T-cell receptor avidity for certain pentamer/MHC complexes might have decreased the sensitivity of our flow cytometric test method, as highlighted by Nepom.[51] However, positive reactions, representing the meaningful and significant findings of this analysis, are not affected by this phenomenon. Table 7 shows the high frequency of positive reactions with two different HLA-A*02:01 pentamers in HLA-A2$^+$ patients, of whom 96% could be expected to express the allele HLA-A*02:01.[52] Should HLA identity of pentamers with patient HLA play a role in the selection of pentamer-specific CD8$^+$ lymphocytes, we would expect that would increase the stringency of our detection system because only T-cell receptors with high affinity for the HIV peptide would bind to the pentamer. Positive reactions in an HLA incompatible setting indicate that the binding affinity to the HIV peptide is exquisitely strong. The constellation might be similar to the direct recognition of incompatible donor HLA peptide by recipient T lymphocytes in organ transplantation.[53]

We were concerned that the selection of $CD8^+$ lymphocytes may have been antigen unspecific. However, the finding that HIV^+ individuals had significantly higher numbers of pentamer-reactive $CD8^+$ lymphocytes than healthy controls strongly suggests that the selected $CD8^+$ lymphocytes were virus specific (Table 1). Mean ± 2 SD of pentamer-reactive $CD8^+$ lymphocytes for the six HIV pentamers in healthy individuals was 0.12%, 0.09%, 0.31%, 0.06%, 0.08%, and 0.12%. This was far below the cutoff level of >0.3% pentamer-reactive $CD8^+$ lymphocytes used in our analysis for the definition of positive test results. The sensitivity of the test could be further improved by investigating more than 100 μL of heparinized whole blood using rare event detection in flow cytometric analysis.

Conclusions

Hemophilia patients living with HIV for ≥30 years showed a broad polyclonal $CD8^+$ T cell response against HIV. The cellular antiretroviral immune response appeared to be strongest and polyclonal during periods of HIV-1 replication and remained detectable in many patients during periods of HIV-1 quiescence. The consistent cellular anti-HIV-1 response in combination with HAART is believed to ensure stability and survival of chronically HIV-1–infected hemophilia patients. HAART might only be efficient in patients with an immune system that is partially functional, similar to antibiotics that are ineffective in patients with severe combined immunodeficiency disease.

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Disclosure Statement

No competing financial interests exist.

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