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Cytotoxic HIV-1 p55^{gag}-Specific CD4$^+$ T Cells Produce HIV-Inhibitory Cytokines and Chemokines.

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CD4$^+$ T-helper cells appear to be essential in sustaining immune responses in chronic viral infections, as the maintenance of CD8$^+$ cytotoxic T-lymphocyte responses and the control of viremia were demonstrated to depend on CD4$^+$ T cell help. In order to investigate the function of HIV-specific CD4$^+$ T cells in chronic HIV-1-infection, 49 chronically HIV-infected patients were analyzed before and 3 and 6 months after initiation of antiviral treatment. Ten patients showed a substantial, although weak, proliferative response to HIV-1-p55^{gag} protein for which no improvement was observed upon initiation of HAART. From one individual, HIV-1-p55^{gag}-specific CD4-positive T-cell clones were generated that were heterogeneous in their TCR Vβ gene usage and HLA-DRB1*13 and DRB1*03 restricted, respectively. In addition, some CD4$^+$ TCC produced substantial amounts of IFN-γ and MIP-1α/β, were perforin-positive, and showed cytotoxic activity. These diverse functional features of HIV-specific CD4$^+$ T cells suggest that they may exert direct antiviral activity.

KEY WORDS: CD4 T cell; HIV; chemokines; cytotoxic; antiviral activity.

INTRODUCTION

Human immunodeficiency virus (HIV) disease is characterized by high viral load in acute infection and HIV infection of CD4$^+$ T cells. Consequently, viral replication in CD4$^+$ T cells leads to a decline of these cells and loss of immune competence. As a result, reduced T-cell proliferative responses to soluble antigens are observed early during HIV infection, which with time and in absence of an antiviral therapy is followed by declining CD4$^+$ T-cell counts and an even greater immune deficiency (1).

Treatment of HIV-positive individuals with highly active antiretroviral therapy (HAART), which combines HIV-1 protease and reverse transcriptase inhibitors, increases the CD4$^+$ T-cell counts and decreases the plasma HIV-1 viral load (2–6). Although the regeneration of peripheral CD4$^+$ T cells by HAART leads to the restoration of CD4$^+$ T-cell proliferative response to recall antigens in chronic HIV-infected patients (5–7), there are no or only weak HIV-gag-specific CD4$^+$ memory T-cell responses detectable in patients with long-term suppression of HIV, if treatment is started in the chronic stage of disease (8, 9). In contrast, individuals controlling viremia in the absence of HIV antiviral therapy and patients who had been put on powerful antiretroviral therapy during primary infection show a strong HIV-1-gag-specific T-helper cell responses (9, 10). These findings suggest that the HIV-specific CD4$^+$ T-cell response is critical for the course of HIV infection.

A number of recent studies revealed that CD4$^+$ T-helper cells play an important role in chronic viral infections by maintaining CD8$^+$ cytotoxic T lymphocytes (CTL) responses and controlling viremia (10, 11). It has been suggested that CD4$^+$ T cells provide direct antiviral effects via production of cytokines such as interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) and may enhance the magnitude and longevity of virus-specific CTL responses (12). Thus

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decreased $CD4^+$ T-cell function in chronic viral infection leads to a loss of antiviral $CD8^+$ CTL function *in vivo* (11, 13–16). However, it seems that the crucial factor in maintaining $CD8^+$ T-cell function during chronic infection is not the total number of $CD4^+$ T cells but rather the presence of antigen-specific T-helper cells (11).

Although it has been shown that $CD4^+$ T cells can have cytotoxic potential and act as CTL (17–27), the mechanisms by which intact HIV-specific T-helper cell functions contribute to control of viremia are not entirely understood. Here, we present a detailed analysis of HIV-1 $p55^{gag}$- and epitope-specific immune response of $CD4^+$ T cells in chronically HIV-infected individuals under HAART to underline the importance of $CD4^+$ T cells in chronic infections. To assess the HIV-$p55^{gag}$-specific response more precisely we generated $p55^{gag}$-specific $CD4^+$ T-cell clones (TCC) and analyzed these in detail for phenotypical and functional features including T-cell receptor usage, HLA-restriction, and their cytotoxic as well as proliferative potential. The present data indicate that $p55^{gag}$-specific $CD4^+$ T cells secrete HIV inhibitory cytokines as well as chemokines and that $p55^{gag}$-specific $CD4^+$ T cells, at least in vitro, can have cytotoxic function. These results suggest that HIV-specific $CD4^+$ cells may exert direct antiviral effector functions by other mechanisms than just providing help for CTL responses.

MATERIALS AND METHODS

Patients

Forty-nine HIV-1-positive, protease-inhibitor naive patients and 10 HIV-negative volunteers were analyzed for their absolute CD4 cell count, HIV-1 RNA, and proliferative response to recall antigen tetanus toxoid (TT) and to $p55^{gag}$. Samples from before and 3 and 6 months after starting HAART were used for these analyses. A consistent and strong $p55^{gag}$ response was obtained by patient F45, a 32-year old female with a CDC stage of A2, who was HIV-infected by heterosexual transmission. From the time of 3 months after initiating HAART we generated 10 $p55^{gag}$-specific T-cell clones (TCC) for further characterization. The HLA-type of this patient was HLA-A 29(19)/A30(19), HLA-B8/B35, HLA-DRB1*03/DRB1*13.

Culture Media

Culture media consisted of RPMI 1640 supplemented with 10% pooled heat-inactivated AB serum (Swiss Red Cross, Bern, Switzerland), 25 mM HEPES buffer, 2 mM L-glutamine (Seromed, Fakola, Basel, Switzerland), 25 μg/ml transferrin (Biotest, Dreireich, Germany), 100 μg/ml streptomycin, and 100 U/ml penicillin. The culture medium CM^+, used to culture TCC and T cell lines, was enriched with 20 U/ml recombinant IL-2 (kindly provided by Dr. A. Cerny Inselspital Bern, Switzerland).

Antigens

The HIV-1-antigen $p55^{gag}$ was generated in recombinant baculoviruses, encoding the entire core protein $p55^{gag}$ (28). Different batches of $p55^{gag}$ were used to confirm specificity. The 25-amino acid-long, overlapping peptides of the $p55^{gag}$ were synthesized based on the HIV-1 PV22 isolate sequence (29). The recall antigen TT was obtained from the Vaccine and Serum Institute, Bern, Switzerland.

Measurement of HIV-1 RNA, $CD4^+$/$CD8^+$ Cell Counts

HIV reverse transcription–polymerase chain reaction (RT-PCR) (Amplicor HIV Monitor, Hoffmann-LaRoche, Basel, Switzerland) was used to quantify the blood HIV-1 RNA concentration in EDTA plasma with a lower detection limit of 100 RNA copies/ml. Phenotyping of $CD4^+$ and $CD8^+$ lymphocytes was performed on fresh EDTA blood samples by two color flow cytometry using an EPICS Profile II flow cytometer (Coulter, Hialeah, FL).

Generation of T-Cell Clones

Peripheral blood mononuclear cells (PBMC) were isolated from 20 ml of heparinized blood samples by density gradient centrifugation using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). PBMC were frozen until use in fetal calf serum supplemented with 10% DMSO and stored in liquid nitrogen. Proliferation of isolated PBMC (1×10^5/well) was measured in a beta counter (Inotech Filter counting system INB 384, Dottikon, Switzerland) 6 days after specific stimulation with $p55^{gag}$ (2 μg/ml) and TT (5 μg/ml) after [^{3}H]-thymidine was added for 6 hr.

For TCC generation, 10^5 PBMC were added in 100 μl/well in a 96 U-bottom tissue culture plate and stimulated with 2 μg/ml $p55^{gag}$ antigen in culture media. CM^+ was added after 7 days of culture. After 13 days, all wells were tested for $p55^{gag}$-specific proliferative response. Specific T cell lines were cloned at day 16 by limiting dilution by seeding 0.5 and 2 cells/100μl per well into a 96-well plate, respectively. For restimulation

Table I. Patients Characteristics[a]

Patients	CDC stage	Pretreatment	Absolute CD4 cell count/μl[b]			HIV-1 RNA (log copies/ml)		
			0	3 (Δ)	6 (Δ)	0	3 (Δ)	6 (Δ)
F34	A1	Naive	789	−41	129	4.2	−2.3	−2.6
F41	A1	Naive	418	42	230	4.7	−2.8	−2.7
F13	A2	Naive	383	−81	−68	5.2	−3.0	−3.3
F45	A2	Naive	350	125	235	4.1	−2.2	−2.2
F1	A2	Naive	324	−105	−62	4.9	−2.6	−3.0
F50	A3	Naive	152	97	200	4.9	−1.8	−1.9
F33	B2	ZDV/3 TC	348	−126	162	3.7	−1.8	−2.2
F49	B3	Naive	59	29	136	5.6	−3.0	−4.0
F46	B3	Naive	98	14	68	5.2	−2.8	−3.6

[a]Clinical characteristics of the ten patients with positive $p55^{gag}$ response.
[b]Months after treatment. Absolute CD4 cell counts/μl at baseline (month 0) and changes (Δ) of cell counts/μl from baseline given. Actual HIV-1 RNA value at baseline and HIV-1 RNA changes (Δ) from baseline are indicated in $\log_{10}$ copies/ml. CDC, Centers for Disease Control.

investigated—0, 3, and 6 months after initiating HAART—five to seven patients presented a proliferative T cell response to $p55^{gag}$ ($3 < SI < 17$, background cpm 77 to 1717). None of 10 healthy HIV-negative control volunteers responded to $p55^{gag}$ (data not shown).

The clinical characteristics of the 10 individuals with positive response to $p55^{gag}$ are shown in Table I. Four of ten patients responded at two or three time points investigated. Three months after the onset of HAART the CD4 cell count increased in 6 of 10 patients and in 8 of 10 individuals after 6 months. Inversely, the HIV-1 RNA dropped in 10 of 10 and in 9 of 10 patients, 3 and 6 months after starting HAART, respectively. No statistically significant differences in CD4 counts were observed when patients responding to P55 were compared to P55 nonresponders either at time 0 ($P = 0.51$) or at 6 month after starting HAART ($P = 0.37$). Similarly, no differences in HIV viral load at time 0 ($P = 0.39$) or at 6 months ($P = 0.74$) were observed between the two groups. Therefore, changes in HIV RNA load and CD4 cell counts during 6 months of HAART were not predictive for the presence of $p55^{gag}$-specific response in T-helper cells.

The proliferative T-cell responses to tetanus toxoid (TT) and $p55^{gag}$ are shown in Fig. 1. Weaker HIV antigen $p55^{gag}$-specific proliferation was seen compared to the TT-specific responses. The median stimulation index (SI) of $p55^{gag}$ in the 10 $p55^{gag}$ responders was 2.83 before HAART. A moderate increase of the median SI up to 4.46 occurred after 6 months of treatment (Fig. 1, upper right), which did not reach statistical significance, however ($P > 0.05$). These data show that relatively low $p55^{gag}$-specific proliferative responses are found in chronically HIV-infected patients before and during HAART. No significant increase in $p55^{gag}$ proliferation and in the number of patients responding to the HIV antigen $p55^{gag}$ under HAART was observed, despite the

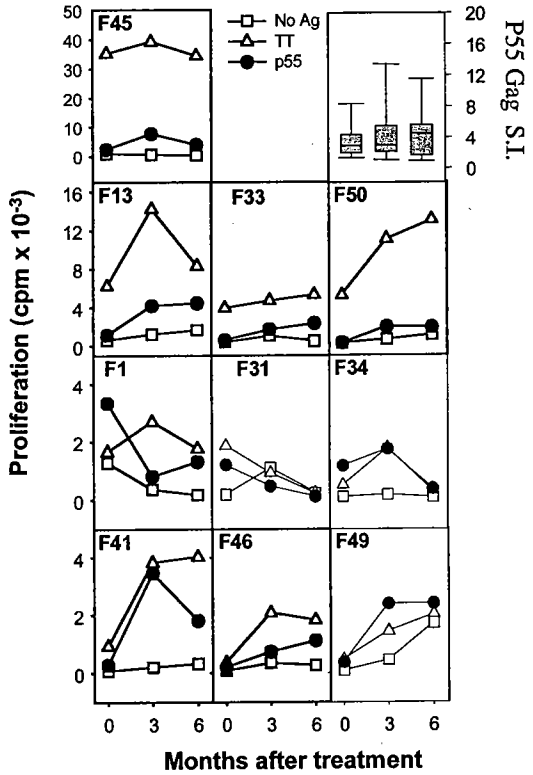

Fig. 1. $p55^{gag}$ and TT-specific proliferation. Proliferation of PBMC from all patients responding to HIV-antigen $p55^{gag}$. PBMC were obtained before and 3 and 6 months after the initiation of HAART. Proliferation was measured against $p55^{gag}$ (2 μg/ml), and tetanus toxoid (TT, 5 μg/ml) and compared to a control without antigen (no Ag). Cpm > 1000 and a stimulation-index (SI) >3 was considered positive in the presence of antigen. Background cpm values in cultures without antigen were for patient F45, time 0, 728;3 months, 478; and month 6,248, respectively. Patients F13 (572, 1195, 1649), F33 (394, 1047, 496), F50 (283, 634, 1127), F1 (1272, 364, 180), F31 (199, 1118, 257), F34 (113, 186, 85), F41 (74, 199, 327), F46 (77, 345, 249), and F49 (85, 439, 1717). Box plots represent the median $p55^{gag}$ responses (SI) per timepoint of all ten patients responding to $p55^{gag}$. Upper bounds represents the 75th and 90th percentiles and lower bounds the 10th and 25th percentiles. Please note different scale.

Table II. p55-Specific Proliferative Response, MHC Restriction, and TCR Vβ Gene Usage

	Autologous PBMC (HLA-DRB1*3/DRB1*13)[a]		Partially matched PBMC (HLA-DRB1*3)[a]		Partially matched PBMC (HLA-DRB1*13)[a]		TCR Vβ[b]
	cpm p55 (no Ag)	SI	cpm p55 (no Ag)	SI	cpm p55 (no Ag)	SI	
Clone 6	18773 (1900)	9.9	582 (129)	4.5	37592 (75)	501.2	n.d.
Clone 9	21554 (951)	22.7	119 (102)	1.2	7553 (107)	70.6	17 + 19[c]
Clone 12	10029 (1894)	5.3	3734 (327)	11.4	993 (508)	1.9	n.d.
Clone 15	9050 (119)	76.1	27732 (152)	182.4	210 (50)	4.2	22
Clone 22	20394 (253)	80.6	35268 (84)	419.9	29 (34)	0.9	22
Clone 25	21210 (387)	54.8	702 (100)	7.0	1570 (78)	20.1	5.1
Clone 26	19098 (270)	70.7	13244 (112)	118.3	353 (80)	4.4	22
Clone 31	9262 (367)	25.2	18589 (64)	290.5	97 (56)	1.7	22
Clone 32	24851 (378)	65.7	14525 (707)	20.5	216 (251)	0.9	n.d.
Clone 36	17701 (643)	27.5	17493 (498)	35.1	365 (215)	1.7	n.d.
Control 17	1831 (1379)	1.3	45 (41)	1.1	21 (49)	0.4	n.d.

[a]TCC (5×10^4/well) were stimulated with or without (no antigen = no Ag) soluble p55^{gag} (2.5 μg/ml) in presence of irradiated autologous (1.5×10^4/well) or partially HLA-DR matched (5×10^4/well) PBMC as APC. Specific proliferation was determinded with a stimulation index (SI) >3 and counts per minute (cpm) > 1000.
[b]TCR Vβ, T-cell receptor Vβ gene usage was analyzed a different panel of 21 different monoclonal antibodies.
[c]Over 90% of clone 9 cells stained double positive for anti-Vβ 17 and anti-Vβ 19, indicating the simultaneous expression of two Vβ elements.

tendency of plasma HIV-RNA to decline and of CD4 cell count to increase over time. Interestingly, p55^{gag}-specific proliferative responses were observed in three individuals with CD4 counts below 200 cells/μl. Overall, no correlation between the p55^{gag} responses and the HIV-1 RNA load or the CD4 cell counts was observed.

Proliferative Response of p55^{gag}-Specific TCC and T-Cell Receptor Vβ Chain Usage

In order to investigate the functionality of virus-specific CD4$^+$ T cell in chronic HIV infection, we generated p55^{gag}-specific TCC from the individual in whom the strongest p55^{gag}-specific proliferative responses were seen (patient F45). All these clones were 100% CD4 positive. Table II shows the proliferative response of 10 p55^{gag}-specific CD4$^+$ TCC and their HLA restriction. One clone, which failed to respond to p55^{gag}, was included as a control clone. Partially HLA-DR-matched PBMC were used to define the MHC restriction of these clones: clones 6, 9, and 25 showed a proliferative response only with HLA-DRB1*13 matched APC, whereas the remaining clones proliferated only with HLA-DRB1*03 matched APC. The lower stimulation indices with the autologous compared to the HLA-DR-matched allogeneic PMBC are likely due to the lower amount of autologous APC, which were available in only limited amount from donor F45.

The p55^{gag}-specific TCC were all CD4 positive (data not shown) and were heterogeneous with regard to T-cell receptor (TCR) Vβ chain usage. Clone 9 cells stained double positive for Vβ17 and Vβ19 when contained, suggesting that two β-chain transcripts are expressed (33).

Epitope Analysis

Proliferation assays using autologous B-LCL as APC and a set of 22 mer peptides overlapping by 15 residues and spanning the entire p55^{gag} protein were performed to define the epitope specificity of the TCC. Epitope analyses for four TCC are shown (Table III). TCC 6 and 25 showed a specificity for two distinct p17^{gag} epitopes (restricted by HLA-DR B1*13), while TCC 12 and 26 were HLA-DR B1*03 restricted and specific for two distinct p24^{gag} epitopes.

p55^{gag}-Specific TCC Produce Cytokines and Chemokines

CD4$^+$ T cells may exert different effector functions besides help and cytotoxicity. To assess these effector functions, we measured cytokine and chemokine production in the p55^{gag}-specific CD4$^+$ T clones with and without antigen-specific stimulation. After p55^{gag}-specific stimulation, 5 of 10 TCC secreted high amounts of IFN-γ and low levels of IL-4 and IL-5 (Table IV), thus representing Th1-like CD4$^+$ T cells. Four clones secreted low levels and one clone (clone 6) intermediate levels of IFN-γ. Clone 32 and 36 mainly secreted IL-4 or IL-5 and therefore might represent Th2-like CD4$^+$ T cells. All tested clones produced TNF-α, regardless of their Th1- or Th2-like cytokine pattern. Moreover, substantial amounts of β-chemokines MIP-1α and MIP-1β

Table III. Overlapping Epitope Analysis.

	B-LCL[a]								
	p55 (no Ag)		$p55^{gag}$-epitope (no Ag)		HLA-DR	Epitope			
	cpm	SI	cpm	SI	restriction	GAG	no	aa #[b]	Epitope sequence
Clone 6	11771 (434)	27.1	39338 (434)	90.6	13	p17 GAG	2	18–42	KIRLRPGGKKKYKLKHIVWASRELE
Clone 25	3059 (630)	4.9	8614 (630)	13.7	13	p17 GAG	3	35–49	VWASRELERFAVNPGLLETSEGCRQ
Clone 12	4158 (334)	12.4	16582 (334)	49.6	3	p24 GAG	13	205–229	INEEAAEWDRVHPVHAGPIAPGQMR
Clone 26	1392 (340)	4.1	1805 (340)	5.3	3	p24 GAG	19	307–331	EQASQEVKNWMTETLLVQNANPDCK
Control 17	493 (617)	0.8	n.d.[c]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

[a]TCC (5×10^4/well) were stimulated with soluble $p55^{gag}$ (2.5 μg/ml) and 22 different overlapping peptides (2.5 μg/ml) in presence of autologous irradiated B-lymphocyte cell lines (B-LCL, 5×10^3/well) for 48 h. Proliferation was determined by [^{3}H]thymidine incorporation. The epitope analysis for 4 TCC is shown.
[b]aa #, Amino acid sequence number.
[c]n.d., Not detectable.

were secreted by the TCC upon $p55^{gag}$-specific stimulation. Compared to the Th2-like cells, the Th1-like clones produced higher amounts of the CCR5-chemokine receptor binding chemokines MIP-1α and MIP-1β. One representative experiment is shown out of three repeats with the same outcome.

$p55^{gag}$-Specific $CD4^+$ TCC Have Cytotoxic Potential for $p55^{gag}$ or $p55^{gag}$ Peptide Pulsed B LCL

Since T-cell-mediated cytotoxicity, at least by CD8 T cells, is considered an important mechanism in viral defense, we investigated the cytotoxic potential of the $CD4^+$ TCC by chromium release assay. As shown in Fig. 2, clone 6, 12, and 25 had cytotoxic activity for $p55^{gag}$ and $p55^{gag}$ epitope 2, 3, or 13 pulsed B-LCL, respectively. The extend of cytotoxic activity observed was comparable to what has been reported for $CD8^+$ CTL (6, 24, 25, 27) and (Fig. 3).

Five of 10 $p55^{gag}$-specific $CD4^+$ TCC were able to lyse $p55^{gag}$-antigen-pulsed, autologous APC (Fig. 3). As described, three of these clones were further able to lyse $p55^{gag}$-epitope- pulsed APC. The remaining TCC were not cytotoxic (<15% specific lysis above background). To analyze the mechanism of cell lysis, blocking experiments with Concanamycin A (CMA) or Brefeldin A (BFA) were performed to distinguish between perforin- and Fas-based cytotoxic pathway. The highly cytotoxic clone 6 was mainly inhibited by CMA, indicating a perforin-mediated cytotoxicity. The remaining four cytotoxic $CD4^+$ TCC were less cytotoxic and both perforin- and Fas-mediated cell lysis appeared to be involved. To confirm the presence of perforin in the $CD4^+$ TCC, immunohistochemical stainings with specific antibodies were performed. Compared to the isotype-matched negative control, an intense granular staining for perforin and granzyme B was observed in the cytoplasm of the $p55^{gag}$-specific $CD4^+$ TCC (Fig. 4a,b:

Table IV. Cytokine and Chemokine Production

	Cytokines (pg/ml)[a]				Chemokines (pg/ml)[a]	
	IFN-γ	IL-4	IL-5	TNF-α	MIP-1α	MIP-1β
Clone 22	11,837	58	n.t.	n.t.	540	2,030
Clone 31	8,142	55	<8	3,380	310	1,320
Clone 15	7,494	26	<8	3,804	370	1,680
Clone 26	3,361	87	414	6,828	350	1,200
Clone 9	3,310	<0.5	<8	963	1,130	2,390
Clone 6	741	12	28	2,313	1,460	2,070
Clone 25	82	<0.5	<8	<62	430	860
Clone 36	45	552	1,414	1,069	210	50
Clone 32	42	647	n.t.	n.t.	40	70
Clone 12	21	<0.5	n.t.[b]	n.t.	150	550
Control 17	41	<0.5	<8	<62	150	30

[a]Cytokine and chemokine pattern of the $p55^{gag}$-specific TCC were measured by ELISA standard assays. Supernatants from proliferation assays with $p55^{gag}$ stimulation were analyzed after 24 h. Results are shown as pg/ml after substraction of media background. TCC 17 was used for SI calculation and did not exeed a SI limit of 1.3 in proliferation assay and was used as control clone.
[b]n.t., Not tested.

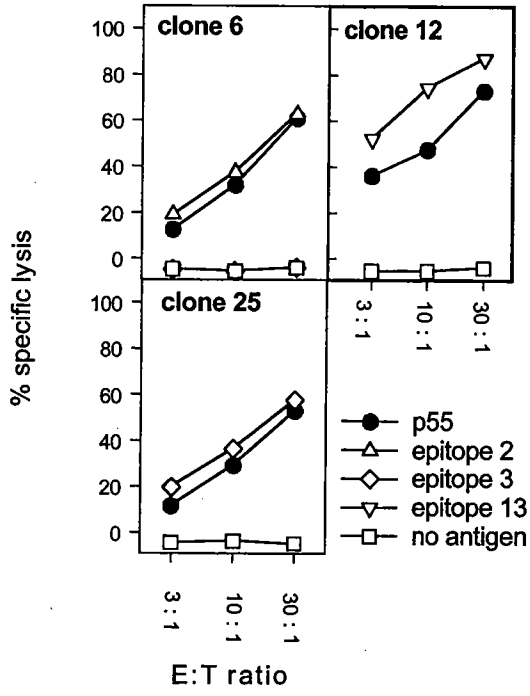

Fig. 2. CTL response against different overlapping epitopes and p55^{gag}. Cytotoxicity of CD$^+$ TCC was measured by chromium release of ^{51}Cr-labeled B-LCL target cells in a 4-hr assay. Autologous B-LCL were pulsed overnight with 10 μg/ml p55^{gag} or individual, overlapping p55^{gag}-peptides. Each of the three clones revealed a cytotoxic activity against p55^{gag} and against one of three p55^{gag}-derived peptides. Spontaneous ^{51}Cr release was always below 21%. Target cells without p55^{gag} or peptide pulsing were not killed (0 – <6%). These values were subtracted from the data shown.

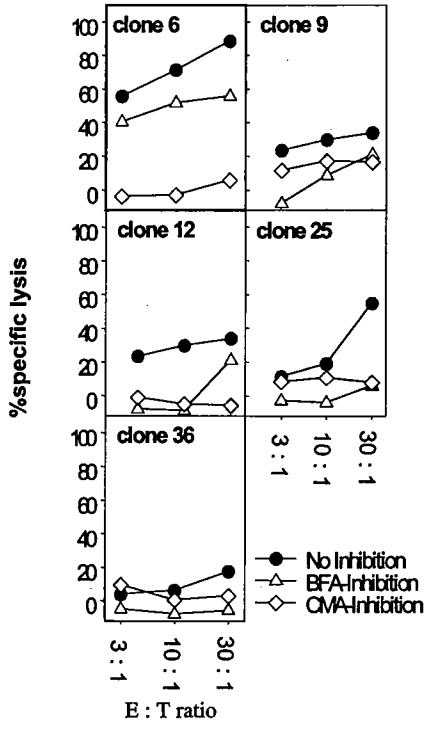

Fig. 3. CTL response: Inhibition by Brefeldin A and Concanamycin A. In a 6-hr assay cytotoxicity of CD4$^+$ TCC was measured by chromium release of ^{51}Cr-labeled B-LCL target cells. Brefeldin A (BFA, 10 μM) was used to block Fas-based pathway and Concanamycin A (CMA, 100 nM) to inhibit perforin-mediated cytotoxicity. APC were pulsed overnight with 10μg/ml p55^{gag} before ^{51}Cr labeling. Results from one representive experiment are shown.

one representative clone is shown). Efforts to stain for Fas-L were unsuccessful, while Fas was detectable predominantly associated with the cell membrane (Fig. 4c).

DISCUSSION

A number of studies have revealed that CD4$^+$ T-helper cells play an important role in chronic viral infection by maintaining CD8$^+$ CTL responses (10, 12, 34). In HIV infection, qualitative and quantitative impairments of the CD4$^+$ T cells occur, which might be relevant for the deleterious natural course of this disease. Interestingly, HAART seems to reconstitute the CD4-mediated immune response to recall antigens but not to HIV-related antigens itself if instituted late in chronic infection (6, 8, 9). To demonstrate the importance of CD4$^+$ T cells in chronic infections next to T-cell help, we analyzed more precisely HIV-1 p55^{gag}- and epitope-specific immune response of CD4$^+$ T cells in chronically HIV-infected individuals under HAART. We analyzed newly generated p55^{gag}-specific CD4$^+$ T-cell clones for T-cell receptor chain usage as well as cytokine and chemokine production to an extent not reported before.

In our study 20% of chronically HIV-infected patients at CDC stage A1-B3 had a moderate but detectable p55^{gag}-specific CD4$^+$ T-cell response under or even before HAART. While HAART significantly increased the responses to the recall antigen TT, no enhancing effect could be observed on the HIV-p55^{gag}-specific response, despite CD4$^+$ cell increase and HIV-1 viral RNA decrease upon initiation of HAART. These data are in agreement with those of Pitcher *at al.* (8), showing that the HIV-specific-p55^{gag} responder frequency did not strongly correlate with plasma viral load. Rather, the median frequencies of gag-specific CD4$^+$ memory T cells were even reduced under continuous HIV-1 suppression with antiretroviral therapy, despite the increase in overall CD4$^+$ counts (8). The reason of this discrepancy between cellular immune response to recall antigens and to HIV-p55^{gag}-antigen after HAART still remains unclear. A possible explanation might be that the

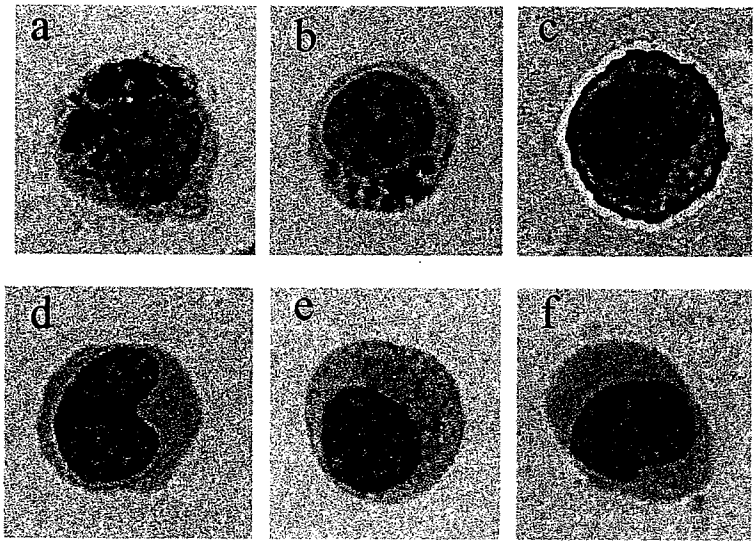

Fig. 4. Immunohistochemical stainings for perforin, granzymes B, and Fas. Immunohistochemical analysis for perforin, granzyme B, and Fas. Strong immunoreactivity for perforin (a) granzyme B (b) and Fas (c) was detected in the $p55^{gag}$-specific $CD4^+$ TCC No. 9. Similar results were obtained for all clones included in Fig. 3. No staining was found using isotype-matched antibodies as negative controls (d–f).

HIV-specific $CD4^+$ T-cell responses are anergized, i.e., due to inadequate antigen presentation by HIV-infected T cells (35, 36).

Despite the only moderate correlation between the progression marker of HIV infection (HIV-1 RNA, CD4 cell count) and the $p55^{gag}$ response in chronically HIV-infected patients, it is still possible and conceivable that $p55^{gag}$-specific responses are functionally important. In long-term nonprogressors and patients treated before seroconversion, Rosenberg *et al.* (10, 37) reported an association between a strong p24-specific proliferative response and reduced plasma viral loads. Moreover, supervised treatment interruption in patients with treated acute infection maintained their virus-specific T-helper cell response (9) and a positive correlation between the levels of p24-specific proliferative responses and the levels of gag-specific $CD8^+$ CTL precursors has been demonstrated in the same cohort (38).

Analysis of the $p55^{gag}$-reactive cells on the clonal level in the present study revealed intriguing functional features. Although $CD4^+$ HIV $p55^{gag}$-specific TCC were generated from one individual only, they were heterogeneous regarding the HLA restriction, the TCR-Vβ gene usage, and the epitope recognition, indicating that this individual developed polyclonal HIV-specific $CD4^+$ T-cell responses. The functional analysis of the clones after antigen-specific stimulation—$p55^{gag}$-protein or peptide—revealed functions suggestive of potent antiviral activities. Five of ten clones secreted high amounts of IFN-γ, which is known to up-regulate cellular immune responses and to render Th1-cells more resistant to HIV infection (1). In addition, the Th1-like $p55^{gag}$-specific TCC secreted the β chemokines MIP-1α and MIP-1β in substantial amounts. This is of particular interest, as MIP-1α and MIP-1β might inhibit the HIV-1 entry into $CD4^+$ T cells by occupying the CCR5 chemokine receptor, which serves as a coreceptor for HIV-1 entry (39–45).

In addition to the production of cytokines and chemokines with potential antiviral effects, five of ten $CD4^+$ T-cell clones showed cytotoxic activity to $p55^{gag}$ or overlapping $p55^{gag}$ peptides-pulsed autologous B-LCL. The cytotoxicity was mediated by both, Fas-L, and perforin-mediated cytotoxic pathways and in one case clearly dominated by perforin (17–24, 27, 46, 47). This is similar to data reported by Yasukawa *et al.* (26), who showed Fas/Fas-Lig-independent cytotoxicity mediated by the granule exocytosis pathway—perforin and granzymes—in human Herpes-simplex-virus-specific $CD4^+$ CTL.

Interestingly, a significant number of our cytotoxic $CD4^+$ T cells did not produce high IFN-γ levels, implying that they are not necessarily Th-1 like T cells. This is in contrast to a study by Hahn *et al.* (20) who report $CD4^+$ CTL to belong to the T-helper type 1 (Th1) subset.

In conclusion, HIV-specific CD4$^+$ T cells appear to exert direct antiviral activity in addition to their traditional role of providing help to and maintaining CD8$^+$ CTL. It might be important to find ways to boost this HIV-specific CD4$^+$ T-cell immune response in chronic HIV-infected patients *in vivo*. Thus, the induction of HLA class II restricted T-helper cell responses by, i.e. an epitope-based HIV vaccine or by therapeutic vaccination with proteins under the cover of HAART may be important not only for providing help to CD8$^+$ CTL but also for employing the diverse antiviral effector function exerted by these cells.

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