

Control of M184V HIV-1 mutants by CD8 T-cell responses

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Abstract Antiretroviral treatment directed against HIV is highly effective, yet limited by drug resistance mutations. We hypothesized that CD8 T cells targeting drug-resistant HIV mutants are able to inhibit viral replication in the setting of a failing therapeutic regimen. We evaluated CD8 T-cell responses and mapped epitopes in HIV-infected patients by interferon-gamma Elispot and intracellular cytokine staining. Autologous virus was sequenced by RT-PCR. Viral replication inhibition assays were performed using M184V mutant virus and CD8 T cell lines. CD8 T-cell responses toward the regions of viral drug resistance mutations in Pol are frequent. Focusing on the M184V mutation, A*02:01-YQYVDDLYV and A*02:01-VIYQYVDDLYV were identified as optimal epitopes for the majority of study subjects. Viral replication of M184V HIV mutants was inhibited by CD8 T cell lines in vitro. In case of a failing lamivudine/emtricitabine containing regimen, individuals with a CD8 T-cell response toward M184V had a significant lower viral load than those without a CD8 response ($p = 0.005$). Two study subjects even achieved an undetectable viral load. Our data suggest that control of M184V mutant virus by CD8 T-cell responses is possible in vitro and in vivo. This control has important implications for therapeutic vaccination strategies.

Keywords HIV · Drug resistance · CD8 T cells · M184V

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Introduction

HIV infection and AIDS are a major global health problem that will probably only be solved with the development of an effective vaccine. So far the efforts for a protective vaccine have been in vain because HIV effectively evades immune pressure [1]. Immunological control of HIV-1 infection is incompletely understood. It has been shown that virus-specific CD8 T-cell responses are responsible for the initial control of high viremia in acute infection [2]. However, HIV-specific CD8 T-cell responses seem to lose their effectiveness during the disease course resulting in increasing viral loads and declining CD4 counts [3, 4].

The control of HIV viremia by a combination of antiretroviral drugs in the chronic phase of infection is well established. The mortality of HIV infection has rapidly declined since the introduction of highly active antiretroviral treatment (HAART). One limitation of its long-term success, however, is the development of viral drug resistance mutations (DRM) as it directly leads to the use of more complex and more expensive treatment regimens. Due to financial constraints, countries in the developing world especially struggle with the emergence of drug-resistant viruses.

A combination of immunologic and pharmacologic control of HIV-1 replication could permit longer applications of simple drug regimens. One hypothesis is that the development of a DRM (e.g. M184V) would not result in rising viral loads if an effective CD8 T-cell response targeting this DRM is in place. In this case, the continued application of lamivudine/emtricitabine keeps the wild-type virus in check and drives it toward M184V, whereas the CD8 T-cell response keeps the M184V virus in check and drives it toward wild-type virus.

It has been shown that strong T-cell responses in general are associated with the maintenance of low viral loads in

patients with drug-resistant HIV-1 [5, 6] and that antiretroviral drugs and T-cell responses work synergistically in controlling viremia in SIV-infected macaques [7–9]. Moreover, peptides containing drug resistance mutations can be recognized by CD8 T cells of infected patients [10–16]. However, fine-mapping of epitopes containing DRM and their HLA restriction is inconclusive [11, 14] and evidence concerning effectiveness of these responses recognizing resistant viruses in humans has not been established. To fill this gap, we analyzed CD8 T-cell responses toward DRM in a cohort of HIV-1 infected individuals. Epitope-specific CD8 T cell lines were used for viral replication inhibition assays *in vitro*. Furthermore, we sought data that provide evidence for the control of the M184V mutants by CD8 T-cell responses *in vivo*.

Materials and methods

Study subjects and HLA typing

Hundred and nineteen HIV-1 infected individuals participated in the study after signing informed consent. The study was approved by the Institutional Review Board of the Ludwig-Maximilians-University, Munich. All experiments were performed in compliance with relevant laws and institutional guidelines and in accordance with the ethical standards of the Declaration of Helsinki.

For the initial screening of CD8 T-cell responses against the viral protein Pol, two groups of subjects were recruited: (1) treatment-naïve individuals who had never taken HAART, irrespective of CD4 count and viral load ($n = 74$) and (2) treatment-failure patients who were taking HAART and had genotypic drug resistance mutations—also irrespective of CD4 count, viral load or DRM ($n = 35$).

Of the 35 patients in the treatment-failure group, 22 patients had a failing HAART containing lamivudine or emtricitabine and were therefore also used for the comparison of maximum viral loads for Fig. 4. Another ten patients were added to this group in order to increase numbers (overall $n = 32$). All 32 study patients had a failing HAART containing lamivudine or emtricitabine with rising viral load and all 32 study patients had a M184V mutation in the autologous virus.

For all study subjects whom we fine-mapped, CD8 T-cell epitopes for high-resolution HLA typing from extracted DNA was performed by the department for transfusion medicine of the University of Munich.

Peptides

Synthetic overlapping peptides were used for screening (Pol: HIV-1 consensus sequence of 2001 either wild-type

or containing DRMs as indicated; 16–19 aa long, 10 aa overlap; Biotrend, Köln, Germany). Truncations of longer peptides were ordered as indicated (NIBSC, England; Biotrend, Köln, Germany; EZBiolab, Carmel, USA).

Interferon-gamma elispot

HIV-specific CD8 T-cell responses were quantified by Elispot assay using fresh or frozen PBMC ($0.5\text{--}1 \times 10^5$ per well) and peptides (final concentration: $12.5 \mu\text{g/ml}$), as described previously [3]. Gamma interferon-producing cells were counted by direct visualization on an AID Elispot Reader (Autoimmun Diagnostika GmbH, Strassberg, Germany) and are expressed as spot-forming cells (SFC) per 10^6 PBMC. Negative controls were ≤ 5 spots. Wells were counted as positive if they were ≥ 50 SFC/ 10^6 PBMC.

For peptide titration assays, peptides were used at concentrations of $12.5\text{--}1.25 \times 10^{-4} \mu\text{g/ml}$ using tenfold dilutions. Peptide comparisons were done in ≥ 2 independent experiments.

Sequencing of autologous virus with viral RNA or proviral DNA

Viral RNA was extracted from the patients' plasma with the QIAamp RNA Viral Mini Kit (Qiagen, Hilden, Germany), or viral DNA was extracted from PBMC by using the Puregene DNA Isolation Kit (Qiagen). Viral RNA was transcribed into cDNA by using the SuperScript II First-Strand Synthesis System for reverse transcriptase PCR (Invitrogen, Darmstadt, Germany) and specific primers. DNA was amplified using nested PCR. PCR cycling conditions were as follows: 97°C for 30 s, 32 cycles of 10 s at 97°C , 30 s at $56\text{--}60^\circ\text{C}$, 40 s at 72°C , and a final extension of 72°C for 10 min. PCR products were sequenced bidirectionally by Eurofins MWG, Martinsried, Germany. Chromas (Technelysium Pty Ltd., Shannon, Ireland) was used to edit and VNTI Align X (Invitrogen) to align sequences.

Cell lines

EBV-transformed B lymphoblastoid cell lines (BCL) were established in R20 medium (RPMI 1640; PAA, Pasching, Austria) supplemented with 2 mM L-glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin, 10 mM HEPES and 20% heat-inactivated FCS (PAA). Antigen-specific CD8 T cell lines were generated from PBMC stimulated with synthetic peptide pulsed HLA-matched BLCL in the presence of 20 million irradiated feeder cells in R10 medium (RPMI 1640 supplemented with 2 mM L-glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin, 10 mM HEPES and 10% heat-inactivated FCS) supplemented with 17 IU/ml of recombinant IL-2 (ImmunoTools GmbH, Friesoythe, Germany).

Intracellular cytokine staining for interferon-gamma production

For determination of HLA class I restriction of CD8 T-cell responses, intracellular cytokine staining assays using autologous or partly matched BLCLs were performed as described [17]. Recognition of M184V mutant viruses was tested by incubating CD8 T cell lines with HLA-A*02:01+ CD4 T cells from an uninfected donor infected with HIV-1 HXB2-M184V and subsequent equal protocol for intracellular cytokine staining for interferon-gamma. Cells were analyzed using a FACS Calibur Flow Cytometer (BD Biosciences, Heidelberg, Germany) and FlowJo (Tree Star Inc., Ashland, USA) software. For negative controls, cells were incubated with BLCLs without peptide or CD4 T cells not infected with HIV-1 HXB2-M184V, but were otherwise treated identically. A positive response was defined as at least threefold above background. Assays were done in ≥ 2 independent experiments.

Viral replication inhibition assay

H9 cells were infected with HIV-1 HXB2-M184V virus (ARP145, NIBSC, Herts, UK) and cultivated at 37°C, 5% CO₂ for 3 weeks. Cell-free supernatant of the virus culture was harvested after 3 weeks and frozen at -80°C until use. The viral titer [50% tissue culture infective doses/ml (TCID₅₀)] was determined by titration at tenfold dilutions of supernatant with H9 cells plated on a 24-well plate. After 2–3 weeks, syncytia-forming H9 cells could be observed, and the titer was calculated depending on the respective dilution factor.

Inocula of 5,000 TCID₅₀ of HIV-1 HXB2-184 V virus were used to infect 5×10^5 HLA-A*02:01+ primary CD4 cells (MOI = 0.01) from an uninfected donor on day 7 after stimulation with the bispecific antibody CD3/CD8. These were cultured with HIV-1-specific CTL lines at E/T ratios 4:1 in duplicate in 24-well plates in 2 ml. All assays were performed in R10 supplemented with 17 IU/ml rIL-2. Half of the supernatant was harvested for quantitative HIV-1 p24 antigen capture ELISA (Perkin Elmer, Rodgau, Germany) and replenished with fresh medium at the indicated time points after infection. Assays were done four times.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software, Version 5.0, and SAS 9.2. For univariate analysis, Mann–Whitney–Wilcoxon and Chi-Square or Fisher's exact tests were used, as appropriate. Tests were two-tailed, and a *p* value less than 0.05 was considered significant. For multivariate analyses, logistic regression models were built assessing for collinearity, significant two-way interaction, and relevant confounding of variables.

Results

CD8 T-cell responses toward regions of DRM within Pol are common

It is known that CD8 T-cell responses toward Pol are broad and exist in up to 92% of untreated subjects even in progressive HIV-1 infection [3, 18–21]. We screened 74 treatment-naïve subjects for CD8 T-cell responses against the viral proteins protease, reverse transcriptase (RT) and integrase in interferon-gamma Elispot and confirmed the high rate of responsiveness to wild-type Pol in untreated individuals (Fig. 1a). Furthermore, we analyzed a cohort of subjects (*n* = 35) experiencing antiretroviral treatment failure. These subjects were screened for CD8 T-cell responses toward wild-type Pol while still on the failing drug regimen presenting with a detectable viral load. Although some of the patients in the treatment failure group were in an extreme salvage situation for their antiretrovirals and presented with a low CD4 count, we were able to detect frequent CD8 T-cell responses, especially within RT (Fig. 1b).

We were mainly interested in the overlap of CD8 T-cell responses and the regions of DRM. As shown in Fig. 1, there were frequent CD8 T-cell responses detectable for the regions of many of the known DRM in both study groups.

For further studies, we concentrated on the DRM M184V that causes complete resistance for lamivudine and emtricitabine. The reasons for choosing this mutation were the following: (1) It is the most common DRM in several studies [22–24]. (2) Lamivudine/emtricitabine are frequently used antiretroviral compounds and are available in developing countries (UNAIDS). (3) In our cohort, the cells of many subjects recognized this region (Fig. 1).

Optimal CD8 T-cell epitopes containing M184V

One of the previously described CD8 T-cell epitopes in the M184 region of RT is the HLA-A*02 restricted VIYQYMDDL (A*02-VL9; bold print = position 184 of RT) [13]. Of all 118 subjects tested for the project (treatment-naïve or treatment failure group), 42 individuals had a CD8 T-cell response toward the overlapping peptide containing the position M184. Only 2 (5%) of these study subjects recognized the A*02-VL9 epitope and stimulating PBMC with the mutated version VIYQYVDDL abrogated the recognition. The M → V mutation thus seems to allow escape from the drug as well as this CD8 T-cell response.

Samri et al. predicted another A*02-restricted epitope to be YQYMDDL_{YV} (A*02-YV9) in this region by a theoretical analysis based on computer scoring [15, 25]. Experimental fine-mapping for this epitope has not been performed so far. We mapped this YV9 epitope as optimal epitope using peptide truncations and confirmed the

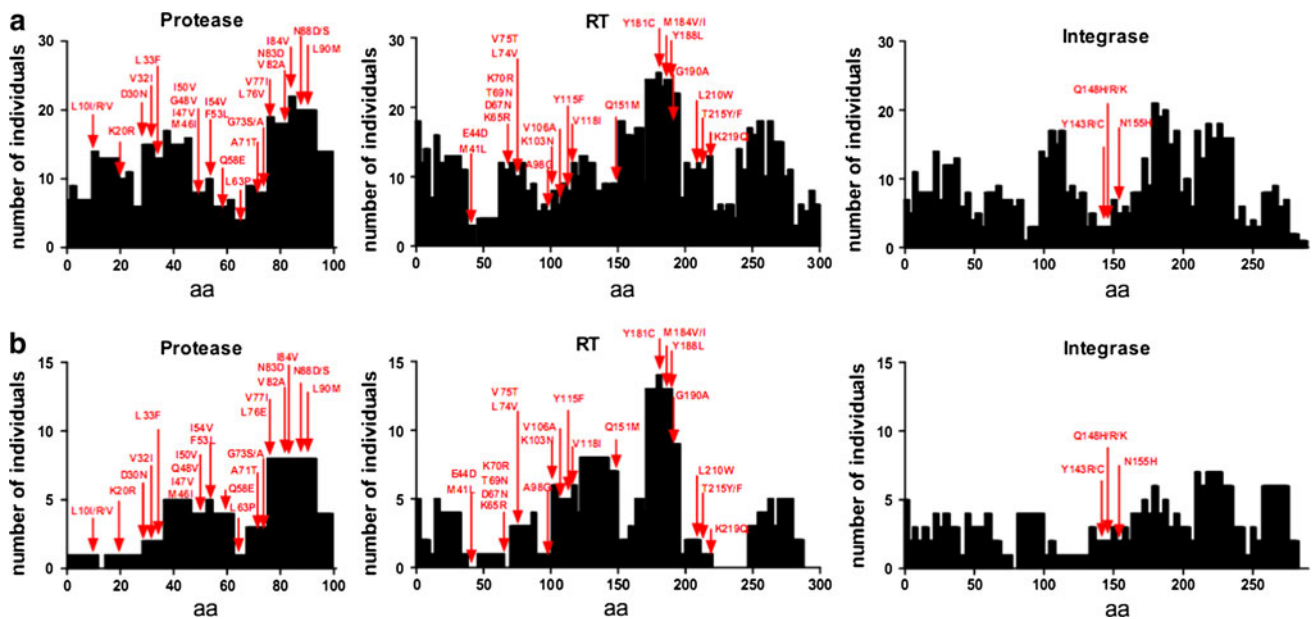


Fig. 1 CD8 T-cell responses toward 16–19mer overlapping peptides spanning HIV protease, reverse transcriptase, and integrase of **a** 74 treatment-naïve subjects and **b** 35 antiretroviral treatment-failure

patients. Indicated in *red* are common drug resistance mutations. On the x axis, each amino acid of the respective protein is listed; on the y axis number of subjects recognizing the amino acid is shown

HLA-A*02:01 restriction in ICS assays (examples in Fig. 2a, b). It was the optimal epitope in 26 of the 42 subjects (62%) in our cohort that had recognized the corresponding overlapping 18-mer peptide. As shown in Fig. 2a, the wild-type epitope YQYMDDLIV was recognized best in treatment-naïve subjects. However, they all still recognized the mutated version YQYVDDLIV, in part with high functional avidity. In contrast, the mutated peptide YQYVDDLIV was the optimal epitope in all lamivudine/emtricitabine experienced individuals whose virus carried the M184V mutation (Fig. 2b). In this group, the wild-type epitope was recognized as well—with different functional avidity as shown in two examples (Fig. 2b).

Three HLA-A*02+ subjects with responses toward the overlapping peptide weakly recognized the A*02-YV9 epitope. Titration assays with truncated peptides revealed VIYQYMDDLIV/VIYQYVDDLIV, likewise restricted by HLA-A*02:01, to be the optimal epitope in these individuals (Fig. 2c, d). For this novel A*02-VV11 epitope, we observed the same effect of the viral M184V mutation as for the YV9 epitope: one treatment-naïve subject with wild-type virus recognized the wild-type epitope best (Fig. 2c), whereas the two treatment-failure patients recognized the mutated peptide best (Fig. 2d).

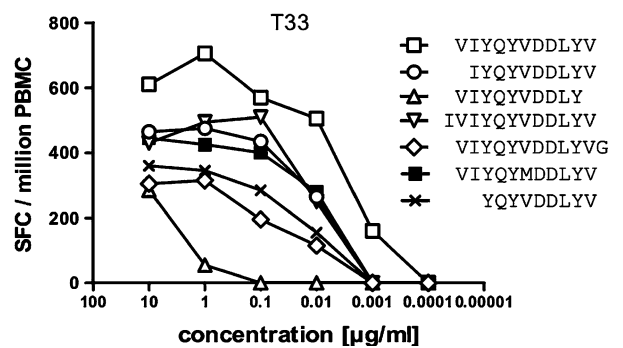
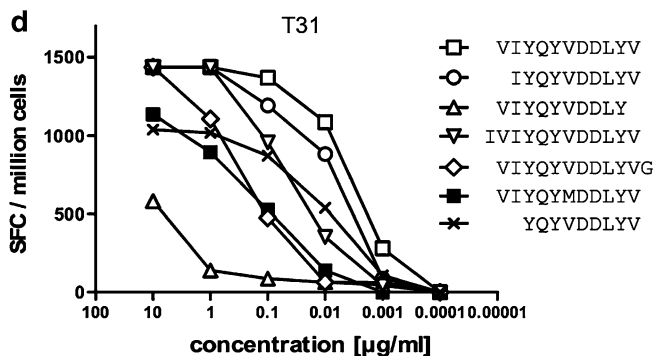
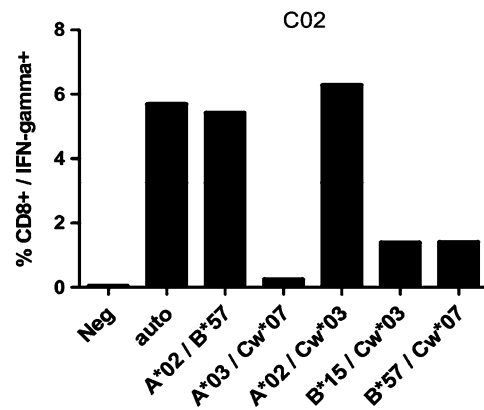
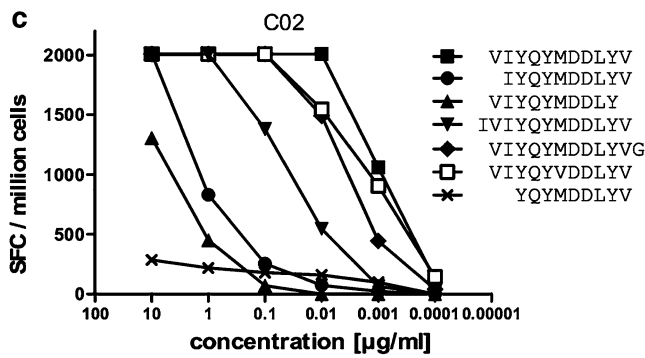
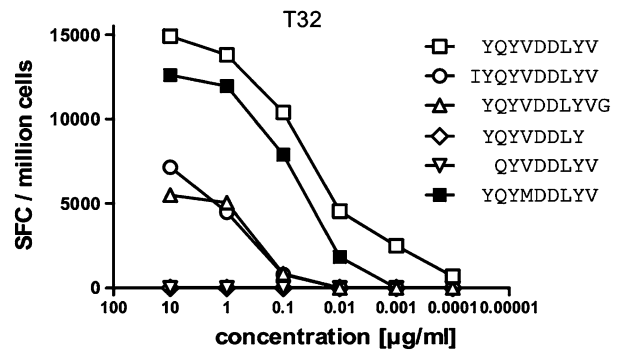
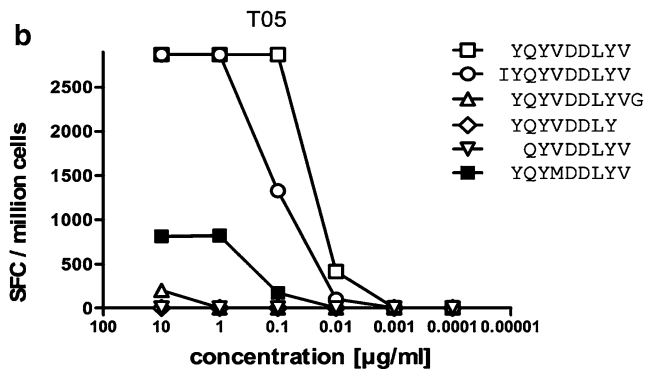
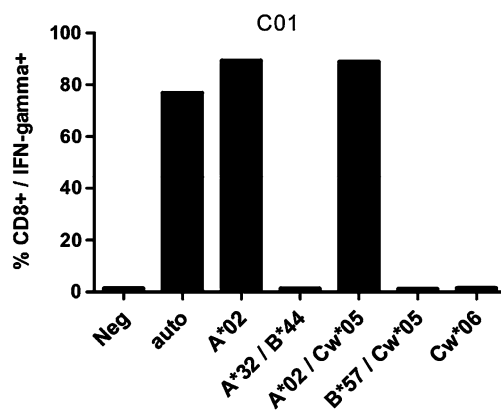
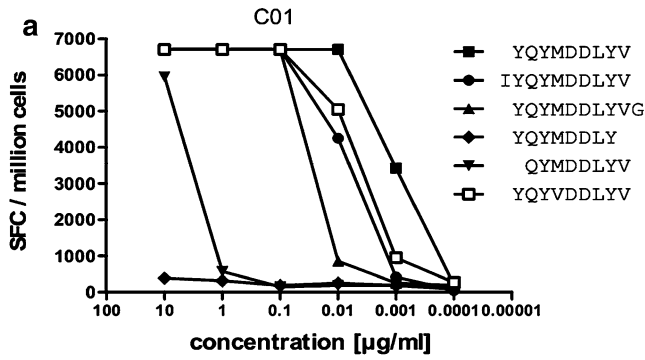
Two of the 42 study subjects with sufficiently strong CD8 T-cell responses for epitope mapping toward the M184V region did not carry the HLA-A*02 allele. Mapping of the respective optimal CD8 T-cell epitopes has not been done so far.

Therefore, YQYMDDLIV/YQYVDDLIV (YV9) and VIYQYMDDLIV/VIYQYVDDLIV (VV11) were the relevant CD8 T-cell epitopes for M184V mutated viruses in our cohort of HLA-A*02:01 positive patients.

M184V mutant viruses are recognized by YQYVDDLIV-specific CD8 T cell lines in vitro

We next wanted to investigate whether CD8 T cells toward the newly defined epitopes could control M184V mutants. To test this hypothesis, we grew CD8 T cell lines specific for the epitope YQYVDDLIV from two study subjects

Fig. 2 Epitope mapping and HLA restriction of CD8 T-cell responses against the region of M184 in the protein reverse transcriptase. **a** Titration of truncated peptides and HLA-A*02 restriction of the epitope YQYMDDLIV with a CD8 T cell line of subject C01 (HLA-A*02:01, -A*32:01, -B*44:02, -B*57:01, -C*05:01, -C*06:02). Example for an untreated individual. **b** Titrations of truncated peptides for the epitope YQYVDDLIV. Examples of two treatment-failure patients recognizing the wild-type epitope with different functional avidity (T05—HLA-A*02:01, -A*02:01, -B*08:01, -B*13:02, -C*06:02, -C*07:02; T32—HLA-A*02:01, -A*25:01, -B*44:02, -B*44:02, -C*05:01, -C*07:04). **c** Titration of truncated peptides and HLA-A*02 restriction of the epitope VIYQYMDDLIV with a CD8 T cell line of subject C02 (untreated individual; HLA-A*02:01, -A*03:01, -B*15:01, -B*57:03, -C*03:04, -C*07:01). **d** Titrations of truncated peptides for the epitope VIYQYVDDLIV. Examples for the two treatment-failure patients (T31—HLA-A*02:01, -A*02:02, -B*35:01, -B*49:01, -C*04:01, -C*07:01; T33—HLA-A*02:01, -A*02:01, -B*15:01, -B*15:01, -C*03:04, -C*04:01). *SFC* spot forming cells, *Neg* negative control, *auto* autologous B cell line



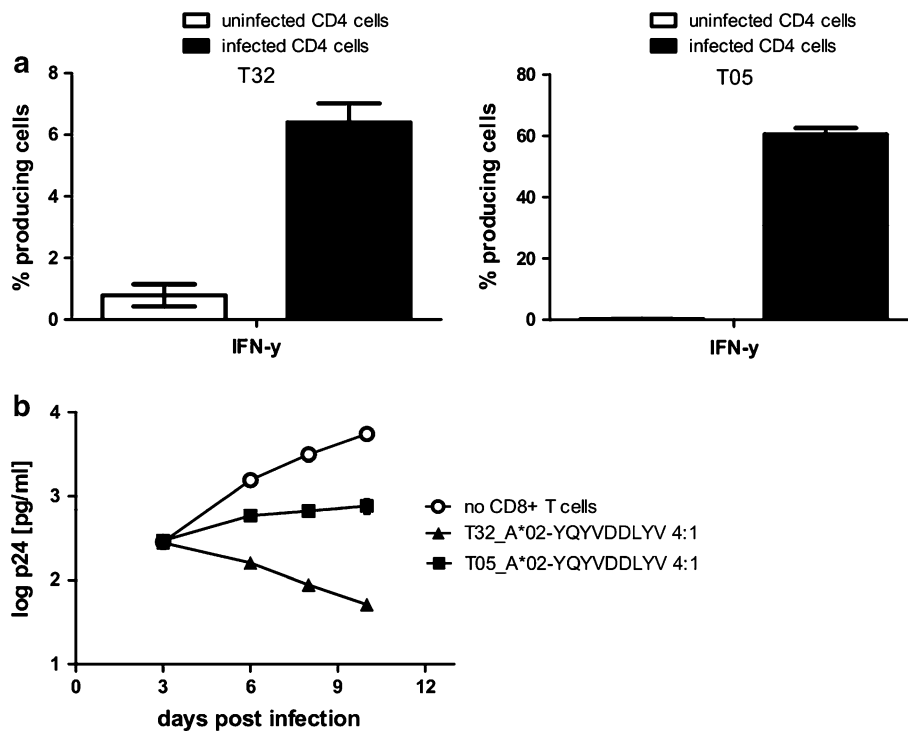


Fig. 3 **a** IFN-gamma (IFN- γ) production of YQYVDDLYV-specific CD8 T cell lines derived from subjects T32 (HLA-A*02:01, -A*25:01; -B*44:02, -B*44:02; -C*05:01, -C*07:04) and T05 (HLA-A*02:01, -A*02:01; -B*08:01, -B*13:02; -C*06:02, -C*07:02) in response to co-culture with HLA-A*02+ CD4 T cells from an uninfected donor (HLA-A*02:01, -A*24:02; -B*27:05, -B*55:01; -C*02:02, -C*03:03) infected with HXB2-M184V. Response of CD8 T cell lines toward

peptide stimulation: T32 = 13%; T05 = 82%. **b** HLA-A*02+ CD4 T cells from the same uninfected donor infected with HXB2-M184V were incubated with or without YQYVDDLYV-specific CD8 T cell lines derived from subjects T32 and T05 with an effector : target cell (E:T) ratio of 4:1. Viral replication was measured by p24 production in culture supernatant. Log p24 logarithmic scale of amount of p24

who were part of the treatment failure group. The autologous virus of both subjects carried the M184V mutation, and the mutated epitope was best recognized in both (data not shown). HLA-A*02:01+ CD4 T cells from an uninfected donor were infected with the HIV-1 HXB2-M184V virus. Infected CD4 T cells were detected by the CD8 effector cells which produced interferon-gamma in response (Fig. 3a). Interferon-gamma production toward infected CD4 T cells was weaker than toward peptide stimulation (T32: 6% vs. 13%; T05: 60% vs. 82%). However, this might be attributed to the antigen excess when adding synthetic peptide.

Viral replication inhibition assays using the same CD8 T cell lines as effectors and HXB2-M184V infected CD4 T cells as targets confirmed recognition of the mutated virus and restriction of viral replication in vitro (Fig. 3b). Interestingly, the inhibiting capacity of the T32 CD8 T cell line was stronger than that of the T05 CD8 T cell line despite the fact that the latter showed a much higher specificity after peptide stimulation. For both CD8 T cell lines, the YQYVDDLYV was the optimal epitope and it was recognized with comparable functional avidity. In addition neither cell line was specific for other HIV epitopes within Gag, Nef or Pol (data not shown).

These data show that CD8 T-cell responses toward YQYVDDLYV can control mutated virus in vitro.

Clinical evidence for control of M184V viruses

To address the question of whether a CD8 T-cell response toward an epitope containing the M184V mutation is associated with viral control in vivo after treatment failure, we first analyzed a cohort of 32 infected patients who had developed antiretroviral treatment failure with a rising viral load and a confirmed M184V mutation while taking a lamivudine or emtricitabine containing regimen (Table 1). Having a CD8 T-cell response toward the M184V containing screening peptide was associated with a tenfold reduction in viral load (median: 4,467 HIV RNA copies/ml versus 45,700 HIV RNA copies/ml; $p = 0.005$; Fig. 4). In addition, having a CD8 T-cell response toward the region M184V was strongly associated with a viral load <10,000 copies/ml (OR 15.0, 95% CI 2.42–93.0, $p = 0.001$). Viral attenuation by the M184V mutation has been shown [26] and could account for lower viral loads. However, the viruses of all 32 subjects included in the analysis—those with CD8 T-cell response and those without CD8 T-cell response—carried the M184V mutation (Table 1). There was a significant

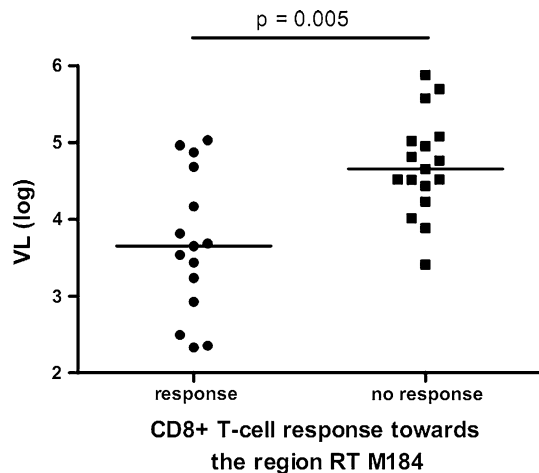


Fig. 4 Comparison of maximum viral loads (VL) of subjects with CD8 T-cell response toward the region of reverse transcriptase containing M184V and without CD8 T-cell response toward this region ($p = 0.005$, Mann–Whitney test). All subjects had developed treatment failure with a rising viral load, and the viruses of all 32 study subjects carried the M184V mutation while receiving a lamivudine/emtricitabine containing regimen (Table 1)

association between CD4 T-cell count and CD8 T-cell response toward the region M184V ($p = 0.02$, Mann–Whitney–Wilcoxon test). However, in multivariate analysis having a CD8 T cell response toward the region M184V remained the only significant predictor for a viral load $<10,000$ copies/ml (aOR 15.44, 95% CI 1.92–124, $p = 0.01$) even after adjusting for genotypic sensitivity score and CD4 T-cell count, which were neither found to be confounders of this association nor significant predictors ($p > 0.81$ in multivariate analysis). Gender and composition of the failing drug regimen (inclusion of protease inhibitors, non-nucleoside reverse transcriptase inhibitors, number of nucleosides/nucleotides) were not significantly associated with viral load, either (all $p > 0.34$ in univariate analysis). In addition, there was no significant difference in CD8 T-cell count of the two study groups ($p = 0.2$): study subjects with CD8 T-cell response had a median CD8 count of $1,048/\mu\text{l}$ (range 287–1,422) and study subjects without CD8 T-cell response had a median CD8 count of $747/\mu\text{l}$ (range 182–1,791).

Therefore, the difference in viral load could be the result of control of the mutated virus by CD8 T cells.

We also analyzed two HIV-infected individuals (T31, T33) with undetectable viral load (<50 copies/ml) who were taking their antiretroviral regimen irregularly (T31: zidovudine/lamivudine/abacavir; T33: zidovudine/lamivudine/abacavir/tenofovir). This was confirmed by pill counting. T31's and T33's PBMC exhibited a robust CD8 T-cell response toward the epitope VIYQYVDDLYV (Figs. 2d, 5d)—interestingly, the 11-mer epitope was the optimal epitope in both individuals. Despite the lack of sufficient drug

intake, T31's viral load was below detection level during the past 6 years with only three exceptions (day 635 till 971; Fig. 5a). Stored plasma samples from these three time points were available, and sequencing of the autologous virus revealed the development of the M184V mutation (Fig. 5b). Nevertheless, the viral load became undetectable again without a change of the treatment regimen. On day 2,310 when the viral load was undetectable, the first PBMC sample was available. After removal of CD8 T cells and antiretroviral drugs, positively selected CD4 T cells readily produced HIV in cell culture. As shown in Fig. 5b, all viral clones that were sequenced from the culture supernatant carried the M184V mutation. T33's viral load was below detection level within the past 2 years with only one exception on day 215 (viral load = 58cp/ml). Plasma samples were not available of this time point. In analogy to T31, virus culture from day 545 showed growth of M184V viruses (Fig. 5b).

In order to exclude other known factors that could explain the subjects' exquisite control of HIV viremia, we analyzed genetic markers associated with control. The HLA genotype was HLA-A*02:01, -A*02:02; -B*35:01, -B*49:01; -C*04:01, -C*07:01 for T31 and HLA-A*02:01, -A*02:01; -B*15:01, -B*15:01; -C*03:04, -C*04:01 for T33. PCR of the CCR5 gene revealed that they did not carry the CCR5 delta32 deletion (data not shown).

Taken together, these findings could indicate that a CD8 T-cell response toward a M184V containing epitope can maintain complete suppression of HIV viremia in the presence of a drug-resistant virus without a change in antiretroviral treatment.

Discussion

Since a protective vaccine against HIV is still out of reach, control of chronic infection by a combination of drug therapy and immunologic means is an intriguing prospect. In this study we investigated the feasibility of this approach with respect to HIV-specific CD8 T cell responses.

We first examined the overlap of CD8 responses and the sites of drug resistance mutations in chronically HIV-infected individuals. We then focused on the M184V lamivudine/emtricitabine resistance mutation. The optimal CD8 epitope was mapped in all study subjects with sufficiently strong responses. The 9-mer YQYMDDLYV/YQYVDLYV was the optimal epitope in the majority of individuals (62% of individuals recognizing the overlapping peptide). This epitope had been predicted by a theoretical analysis using computer scoring [15] but never proven via experimental fine-mapping. The second optimal CD8 T cell epitope which was recognized by only three of the study subjects was the 11-mer VIYQYMDDLYV/VIYQYVDDLYV. This

Table 1 Clinical data of study subjects for Figs. 4 and 5

ID	VL	CD4	CD8	YOD	HAART	RT-DRM	Gender
T01	32,979	535	586	1997	3TC/AZT/NVP	A62V, Y181C, M184V	Female
T02	48,331	366	596	2000	3TC/AZT/ABC	D67N, K70R, M184V , T215I, K219E	Male
T03	7,670	60	441	2003	FTC/TDF/FPV	D67N, M184V	Male
T04	27,300	46	725	2007	FTC/TDF/EFV	69S, L74I, K103N, M184V , 335D, 371V, 399D	Male
T05	92,000	115	1,048	2008	FTC/TDF/EFV	V118I, M184V	Male
T06	758,578	18	109	1995	3TC/TDF/EFV/LPV_r	M141L, D67G, T69SCG, L74V, K101H, V108I, Y181C, M184V , G190A, T215TY	Female
T07	107,152	181	1,201	2000	3TC/AZT/ABC/ATV/RTV	K103N, M184V	Male
T08	120,226	270	821	1988	FTC/TDF/EFV	K20R, M41L, E44D, D67N, 106I, V118I, 135T, M184V , 188L, L210W, T215Y, K219N	Male
T09	90,164	96	1,694	1995	3TC/TDF/EFV/TPV/RTV/T20	M41L, D67N, T69D, K70R, L74I, V75T, V118I, Y81C, M184V/I , G190S, T215F, K219Q	Male
T10	500,001	12	182	1993	FTC/TDF/ATV/RTV	M184V	Male
T11	45,700	120	1,551	1991	FTC/TDF/TPV/RTV/ABC	V75I, F77L, K103N, Y115F, F116Y, V118I, Q151M, M184V , K219E	Male
T12	58,775	90	357	1995	FTC/TDF/LPV_r	M41L, M184V , L210W, T215F	Male
T13	229	147	571	1984	3TC/TDF/LPV_r	M41L, D67N, K103N, M184V , L210W, T215Y	Male
T14	104,713	19	226	1988	FTC/TDF/ETV/DRV/RTV	M41L, D67N, T69N, K70R, L74I, M184V , T215F, K219Q	Male
T15	10,301	407	819	1998	FTC/TDF/EFV	M41L, L74V, K101E, V118I, M184V , Y181C, Y188L, G190S, T215Y	Male
T16	4,854	174	1,422	1985	3TC/ABC/ddI	M41L, E44D, D67N, V118I, M184V , L210W, T215Y, K219R	Male
T17	2,749	137	1,322	2005	FTC/TDF/EFV	K65R, K103N, M184V , P255H	Male
T18	380,189	30	747	1989	3TC/ABC/AZT	M41L, E44A, K67D, A98G, K101H, V118I, Y181C, M184V , G190A, L210W, K219N	Male
T19	33,050	154	908	1999	FTC/TDF/d4T	D67N, K70R, M184V , K219Q	Male
T20	215	240	836	1991	FTC/TDF/NVP	V108I, Y181C, M184V	Male
T21	3,452	311	1,016	1986	3TC/ABC/ATV/RTV	M41L, L74V, V118I, M184V , L210W, T215Y	Male
T22	65,366	230	936	1996	FTC/TDF/NVP/FPV/RTV	D67N, K70R, M184V , T215I, K219E	Male
T23	14,791	399	1,404	1989	3TC/d4T/NFV	D67N, K70R, K101E, M184V , G190A, T215F, K219E	Male
T24	32,801	67	467	1987	FTC/TDF/SQV/ATV/RTV/T20	M41L, D67N, V75I, V118I, M184V , Y188L, L210W, T215Y, K219R	Female
T25	1,728	316	616	1993	3TC/AZT	K70R, M184V , 333E	Female
T26	74,747	376	528	1996	3TC/d4T	M184V	Male
T27	4,467	110	287	1985	3TC/LPV_r/SQV	K65N, K101G, K103N, M184V , G190A, L215W, T215N	Female
T28	6,554	594	1,192	1998	3TC/AZT/NVP	A98G, K101E, Y181C, M184V , G190A	Male
T29	2,576	330	980	1995	3TC/ABC/LPV_r	V179D, M184V	Male
T30	17,000	160	1,791	2008	FTC/TDF/ATV/RTV	V108I, Y181C, M184V	Female
T31	823	376	1,235	1996	3TC/AZT/ABC	M184V	Male
T32	312	346	1,280	1996	3TC/ABC/LPV_r	D67N, K70R, V75T, M184V , K219E	Male

VL viral load, YOD year of diagnosis, HAART highly active antiretroviral therapy, RT-DRM reverse transcriptase drug resistance mutation, 3TC lamivudine, FTC emtricitabine, AZT zidovudine, ABC abacavir sulfate, TDF tenofovir disoproxil fumarate, d4T stavudine, ddI didanosine, NVP nevirapine, EFV efavirenz, ETV etravirine, TPV tipranavir, SQV saquinavir mesylate, RTV ritonavir, LPV_r lopinavir/ritonavir, FPV fosamprenavir, DRV darunavir, ATV atazanavir sulfate, NFV nelfinavir mesylate, T20 enfuvirtide

epitope has not been described so far. Van der Burg et al. had described the 10-mer epitope IYQYMDDLIV as HLA-A*02 restricted epitope [25]. However in our

study subjects, the 11-mer was better recognized than the 10-mer (Fig. 2), even if an 11-mer A*02 epitope is unusual.

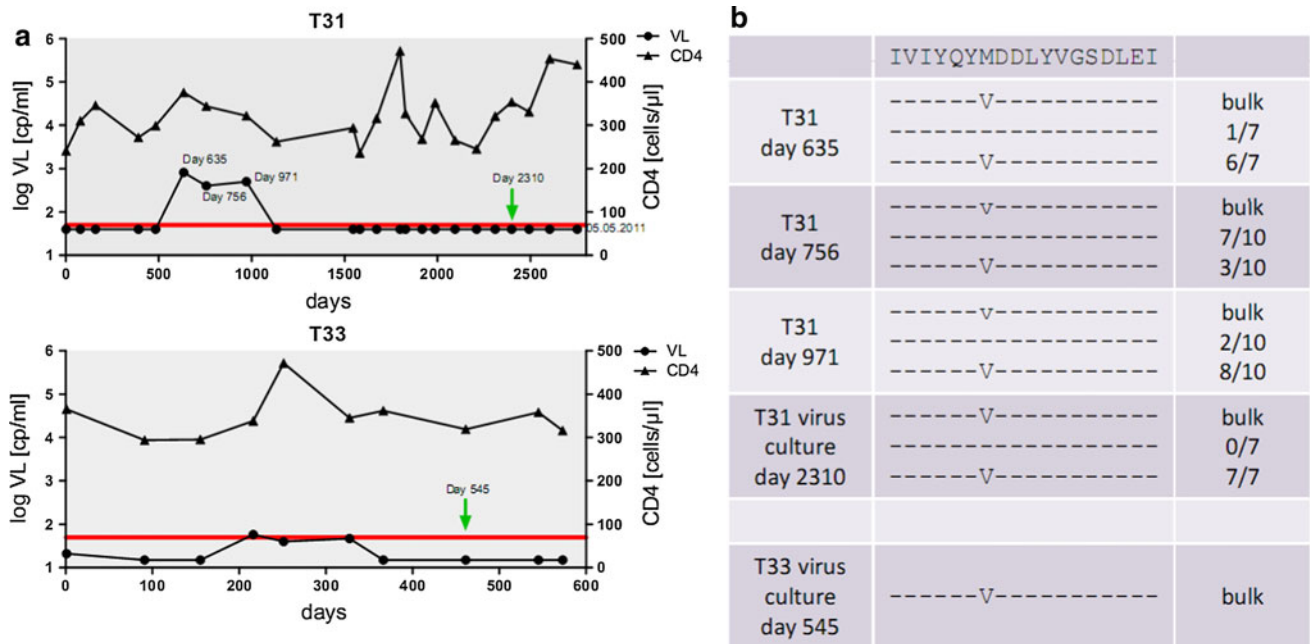


Fig. 5 **a** Course of viral load (VL) and CD4 T-cell count of subjects T31 and T33 who took their antiretroviral treatment irregularly. Cut-off for viral load testing: red line. Time of viral culture: green arrow.

b Bulk and clonal sequencing of the individuals' virus at different time points and from culture supernatant (T31 day 2,310; T33 day 545)

The M184V mutation acted as escape variant for the known CD8 epitope VIYQYMDDL in our cohort which is in accordance with the data of the first publication of this epitope in 1996 [13].

Having defined the CD8 T cell responses recognizing M184V mutated peptides our next question was if these responses also recognize M184V mutant viruses. We show definitive evidence that primary CD8 T cell lines specific for the YQYVDDLIV epitope can inhibit viral replication of M184V containing viruses in vitro.

To address the question of in vivo control of M184V mutant viruses, we identified patients who developed antiretroviral treatment failure with a rising viral load under a lamivudine/emtricitabine containing regimen and with a proven M184V mutation. Having a CD8 T cell response toward the M184V containing overlapping peptide was the only significant independent predictor of a viral load <10,000 copies/ml identifiable in these individuals. As viral load is a strong predictor of disease progression even in the setting of a failing HAART regimen [27], the CD8 T cell response toward M184V can be assumed to be protective. Viral attenuation by the M184V mutation has been shown [26] and could account for lower viral loads. However, the viruses of all 32 patients included in the analysis—those with CD8 T cell response and those without CD8 T cell response—carried the M184V mutation (Table 1). Lower viral loads in treatment failure individuals with overall strong CD8 T cell responses have been described previously [5, 6] and might be partially attributable to specific

CD8 responses targeting DRM. One of these reports shows stronger CD8 T cell responses toward Pol than toward the other HIV proteins in treatment-failure patients and a shift of immunodominance from Gag to Pol [6, 28].

The 32 treatment failure individuals included in this analysis were identified due to a rising viral load above the detection limit of 50 copies/ml in our assay (bDNA). We therefore set out to determine whether responses against M184V may help to maintain an undetectable viral load. The analysis of two study subjects, T31 and T33, showed an undetectable viral load in the setting of a robust CD8 T cell response toward VIYQYVDDLIV and an insufficient antiretroviral drug intake with development of the M184V mutation. We tried to exclude other reasons for control of HIV viremia in these individuals. CD4 T cells of the individuals readily produced M184V mutated HIV in cell culture in the absence of CD8 T cells and antiretroviral drugs proving that T31 and T33 were not infected with a replication incompetent virus. Additionally, they did not carry the genetic factors which have been shown best to induce spontaneous control of HIV viremia, the HLA class I alleles B*27 and B*57 [28]. In contrast T31 carried two HLA class I alleles that are associated with more rapid disease progression, namely HLA-B*35 and HLA-C*07 [29]. We also did not find heterozygosity of the CCR5 delta 32 deletion, shown to slow disease progression of HIV-infected patients, particularly during HAART [30]. This situation in T31/T33 is possibly comparable to one in the animal model. SIVmac251 wildtype infected macaques that were

treated with tenofovir monotherapy developed the K65R mutation but maintained an undetectable viral load in the presence of CD8 T cells [8].

In conclusion we present evidence that a CD8 T cell response toward M184V is beneficial in the context of a failing lamivudine/emtricitabine containing HAART regimen. According to UNAIDS, 99% of first-line regimens in developing countries contain either lamivudine or emtricitabine, whereas only 47% of second-line regimes still contain these drugs. Genotypic resistance testing reveals a prevalence of the M184V mutation in 64–100% of resistant viruses in different populations [22–24]. Therefore, lamivudine and emtricitabine are frequently used drugs, but treatment failure arises quickly. Based on the data presented here, it seems an intriguing idea to design a therapeutic vaccination for HLA-A*02+ patients that induces CD8 T-cell responses against M184V.

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Ethical standards All experiments comply with the current German laws.

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