University of Groningen

The presence of protective cytotoxic T lymphocytes does not correlate with shorter lifespans of productively infected cells in HIV-1 infection

Spits, Hilde B; Mudrikova, Tania; Schellens, Ingrid M M; Wensing, Annemarie M J; Prins, Jan M; Feuth, Thijs; Spierings, Erik; Nijhuis, Monique; van Baarle, Debbie; Borghans, José A M

Published in:
Aids

DOI:
[10.1097/QAD.0000000000000914](https://doi.org/10.1097/QAD.0000000000000914)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2016

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Spits, H. B., Mudrikova, T., Schellens, I. M. M., Wensing, A. M. J., Prins, J. M., Feuth, T., Spierings, E., Nijhuis, M., van Baarle, D., & Borghans, J. A. M. (2016). The presence of protective cytotoxic T lymphocytes does not correlate with shorter lifespans of productively infected cells in HIV-1 infection. *Aids*, 30(1), 9-17. <https://doi.org/10.1097/QAD.0000000000000914>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

The presence of protective cytotoxic T lymphocytes does not correlate with shorter lifespans of productively infected cells in HIV-1 infection

Hilde B. Spits^a, Tania Mudrikova^b, Ingrid M.M. Schellens^c,
Annemarie M.J. Wensing^d, Jan M. Prins^e, Thijs Feuth^b, Erik Spierings^a,
Monique Nijhuis^b, Debbie van Baarle^{a,c} and José A.M. Borghans^a

Objectives and design: CD8⁺ cytotoxic T lymphocytes (CTL) are important in the control of HIV infection. Although CTL are thought to reduce the lifespan of productively infected cells, CD8⁺ T-cell depletion in simian immunodeficiency virus-infected rhesus-macaques showed no effect on the lifespan of productively infected cells. As CD8⁺ T-cell responses that successfully delay HIV disease progression occur only in a minority of HIV-infected individuals, we studied the hypothesis that the ability of CTL to reduce the lifespan of productively infected cells is limited to protective CTL responses only.

Methods: We correlated features of CD8⁺ T cells that are associated with control of HIV infection, namely restriction by protective human leukocyte antigen (HLA) alleles, and/or a broad, high or poly-functional Gag-specific CD8⁺ T-cell response, to the lifespan of productively infected cells in 36 HIV-infected individuals, by measuring their plasma viral load declines immediately after start of combined antiretroviral therapy.

Results: The average lifespan of productively HIV-infected cells varied greatly between individuals, from 1.01 to 3.68 days (median 1.82 days) but was not different between individuals with or without the protective HLA molecules B27 or B57 ($P=0.76$, median 1.94 and 1.79 days, respectively). Although the CD8⁺ T-cell response against HIV Gag was the dominant HIV-specific T-cell response, its magnitude ($r^2=0.02$, $P=0.5$), breadth ($r^2=0.03$, $P=0.4$), and poly-functionality ($r^2=0.01$, $P=0.8$), did not correlate with the lifespan of productively HIV-infected cells.

Conclusion: The features of CD8⁺ T-cell responses that have clearly been associated with control of HIV infection do not correlate with a reduced lifespan of productively infected cells *in vivo*. This suggests that protective CD8⁺ T cells exert their effect on target-cells before onset of productive infection, or via noncytolytic mechanisms.

Copyright © 2015 Wolters Kluwer Health, Inc. All rights reserved.

AIDS 2016, **30**:9–17

Keywords: cytotoxic T cells, disease progression, HIV-1, human leukocyte antigen, productively infected cells, protective, T cells

^aLaboratory of Translational Immunology, ^bDepartment of Internal Medicine and Infectious Diseases, University Medical Center Utrecht, Utrecht, ^cDepartment of Immune Mechanisms, National Institute for Public Health and the Environment (RIVM), Bilthoven, ^dDepartment of Medical Microbiology, Virology, University Medical Center Utrecht, Utrecht, and ^eDepartment of Internal Medicine, Division of Infectious Diseases, Academic Medical Center, Amsterdam, the Netherlands.

Correspondence to José A.M. Borghans, PhD, KC 02.085.2, P.O. Box 8509, 3508 AB UTRECHT, The Netherlands.

Tel: +31 088 75 542 75; e-mail: J.Borghans@umcutrecht.nl

Received: 30 April 2015; revised: 18 September 2015; accepted: 30 September 2015.

DOI:10.1097/QAD.0000000000000914

Introduction

CD8⁺ cytotoxic T lymphocytes (CTL) are thought to play an important role in the control of HIV infection, and current vaccines are developed based on that premise. The importance of CTL in HIV control is suggested by a temporal association between the appearance of HIV-specific CD8⁺ T-cell responses and the decline of viral load during acute infection [1]. More directly, depletion of CD8⁺ T cells in simian immunodeficiency virus (SIV)-infected rhesus-macaques during chronic infection has been shown to cause a dramatic increase in SIV viral load [2,3], and vaccine-induced CTLs were shown to be able to control SIV replication [4]. In HIV-infected humans, the presence of certain human leukocyte antigen (HLA) molecules is associated with the rate of progression to AIDS and viral-load set-point, suggestive of an important role for CTL [5,6]. The latter finding was further corroborated by a world-wide genome-wide association study in long-term nonprogressors (LTNP), where the only significant single nucleotide polymorphisms were found within the peptide-binding pockets of HLA-class I molecules [7].

Not all CD8⁺ T cells have the same impact on HIV-disease progression. CD8⁺ T cells restricted through HLA-B27 and B57 (henceforth referred to as protective CTL), for example, are clearly associated with relatively slow disease progression whereas the presence of HLA-B*35:03 is associated with rapid progression [8–11]. Multiple studies independently found that individuals with slow HIV-1 disease progression tend to make broad and strong CTL responses against HIV-1 Gag, whereas CTL responses of individuals with rapid disease progression and high HIV-1 viral loads are more focused towards epitopes from Env and accessory/regulatory proteins [12–18]. Importantly, even irrespective of their HLA restriction, the number of Gag-specific CTL responses was found to correlate negatively with HIV viral load and with the rate of HIV disease progression [15].

It is important to understand what makes CTL associated with slower HIV-1 disease progression more protective than other CTL. Part of the answer probably lies in the preferential targeting of the p24 Gag protein [19], which is structurally very constrained. As a consequence, viral mutations in these regions tend to lead to severe viral fitness loss. Immune escape mutations in the immunodominant HLA-B27 and B57 restricted epitopes KK10 and TW10 – both part of the p24 Gag protein – for example, have been shown to lead to a dramatic loss in viral fitness [20–22]. Such detrimental mutations will only be selected for, if they outweigh the selective pressure exerted by the specific CTL response. Differences in the quality of CTL responses may therefore also contribute to the different levels of protection conveyed by CTL. Indeed, CD8⁺ T cells of LTNP have been shown to be highly poly-functional, that is they have the capacity to produce a broad spectrum of cytokines [8].

It is generally thought that CTL recognize and kill productively infected cells, and thereby effectively shorten the time during which infected cells produce new viral particles [23]. However, two studies have challenged this belief. In both studies, the CD8⁺ T-cell pool of SIV-infected rhesus-macaques was completely depleted, and its effect on the lifespan of productively SIV-infected cells was measured by estimating the slope of viral decay in the first weeks after the start of successful antiviral treatment [24,25]. Although an increase in viral load could be observed after depletion of the CD8⁺ T cells, most likely due to the role of CD8⁺ T cells in viral control, both studies found no difference between the lifespans of productively infected cells in rhesus-macaques with or without CD8⁺ T cells. The authors concluded that the CD8⁺ T cells did not exert their antiviral effect through the ‘classical’ cytotoxic killing of productively infected cells, but instead may suppress viral replication by noncytolytic mechanisms or via cytolytic killing of infected cells *before* their productive infection.

An alternative explanation for these findings may be that only the most protective CTL responses are capable of shortening the lifespan of productively HIV-infected cells. If only few individuals carry CTL responses that are capable of reducing the lifespan of productively infected cells, their effect may go undetected in studies in which such protective CTL responses are rare or even absent. Here, we investigated whether CTL with features that are known to be associated with slow HIV-1 disease progression – including restriction by one of the protective HLA molecules or a strong, broad and poly-functional CTL response to HIV Gag – are associated with a reduced in-vivo lifespan of productively infected cells in HIV-1-infected humans.

Materials and methods

Patient characteristics

Blood samples were obtained from 36 HIV-1 subtype B infected patients, 20 of whom participated in the THILIHT study, which was designed specifically to correlate immunological parameters with the lifespan of productively infected cells. Before start of combined antiretroviral therapy (cART), a large amount of blood was drawn to perform immunological experiments. During the first week of therapy, blood was sampled at day 0,1,2,4,6 and 7 to accurately determine the slope of the plasma viral-load decline. Study participants received 600 mg efavirenz, 200 mg emtricitabine, and 245 mg tenofovir disoproxil.

The other 16 individuals were part of the ERA study, of which the primary objective was to study the effect of highly suppressive combination antiretroviral treatment on the plasma viral-load decay. Detailed procedures have

been described elsewhere [26]. All participants were antiretroviral-therapy naive before enrolment in this study, except for ERA-005, who received 1 year of zidovudine and lamivudine up to 1 year prior to enrolment. Both studies were approved by the medical ethics committees of the participating centres and written informed consent was obtained from all donors.

The lifespan of productively infected cells

EDTA plasma HIV-1 RNA decay after the initiation of antiretroviral therapy was used to estimate the lifespan of productively infected cells. In the 20 THILIHT participants HIV-1 levels were quantitated by COBAS AmpliPrep/COBAS TaqMan HIV-1 Test(v2.0), with a sensitivity of more than 20 copies/ml, and in the ERA participants as described previously [26] with a final sensitivity of more than 50 copies/ml. Plasma viral loads were measured for 12 weeks after start of therapy. The slope s of the HIV-1 RNA decline during the first 7 days was used to calculate the average lifespan of the productively infected cells as $1/s$, as published previously [27,28].

HLA typing

High-resolution HLA typing was performed for all included participants using sequence-based typing for 2 loci (HLA-A, and HLA-B) according to the manufacturers' guidelines (GenDx, Utrecht, The Netherlands). All allele and genotype ambiguities at the 2-field level were resolved.

Peripheral blood mononuclear cells/blood separation

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood before start of treatment using Ficoll (GE Healthcare Lifesciences, Boston, Massachusetts, USA) density separation, as described previously [29]. PBMCs were isolated, cryopreserved and stored in liquid nitrogen within 24 h of collection. All experiments were performed on previously frozen PBMCs.

Interferon γ enzyme-linked immunospot (ELISpot) assay

Interferon (IFN) γ -producing antigen-specific CD8⁺ T cells were measured using the IFN γ Elispot assay, as described previously [30]. The IFN γ CD8⁺ T-cell response was measured after stimulation with total overlapping peptide pools (15mers with 11 overlap, Consensus B 2007, NIH AIDS Research and Reagent programme) of Env, Nef, Gag or Pol at a concentration of 2 μ g/ml. PHA stimulation served as a positive control. Results were analysed with an AELVIS ELIScan (ELIA-analyse Software v4; A.EL.VIS, Hannover, Germany). The number of IFN γ -producing cells was calculated by subtracting the unstimulated control value. Samples with at least twice the number of spot-forming units (SFU) of the negative control were considered positive.

Matrix-based Elispot assay

Initial screening for T-cell responses was performed using the IFN γ -Elispot assay (as described above) with the peptides in a matrix format [31]. Subtype B Gag-peptide sets (15mers with 11 overlap, Consensus B 2007) were pooled in an 11-by-11 matrix format, with a final concentration of the individual peptides of 2 μ g/ml. The resulting potential epitopes were matched to the epitopes predicted from the Gag Consensus B 2007 reference strain (IEBD analysis resource, <http://tools.immuneepitope.org/processing/>, date:1-Apr-2014) and all Gag epitopes recorded in the Los Alamos database (<http://www.hiv.lanl.gov/content/immunology/maps/ctl/Gag.html>, date:1-Apr-2014). In-vitro confirmation of the positive matrix responses was performed for the 20 participants included in the THILIHT study using the corresponding individual peptides at a concentration of 10 μ g/ml, as depicted in Figure S1, <http://links.lww.com/QAD/A799>. PHA stimulation served as a positive control, and medium served as a negative control.

T-cell phenotype

Phenotype and expression of senescence markers on CD4⁺ and CD8⁺ T cells was measured after extracellular staining with the monoclonal antibodies described in Table S1, <http://links.lww.com/QAD/A798>. All incubations were performed at 4°C (20 min) after which cells were fixed (cellfix; BD, Heidelberg, Germany) and analysed by flow cytometry.

CD8⁺ T-cell stimulation and intracellular cytokine staining

CD8⁺ T-lymphocytes (at 2×10^6 cells/ml) were incubated with 2 mg/ml Gag-peptide pool (see above) and antiCD107a-FITC (BD) for 6 h. As a positive control, PMA and ionomycin (Sigma-Aldrich, The Netherlands; 5 ng/ml and 1 μ g/ml respectively) were used. After 1.5 h, Brefeldin A (3 μ mol/l, BD) and Monensin (2 μ mol/l BD) were added. Surface staining was performed with monoclonal antibodies described in Table S1, <http://links.lww.com/QAD/A798>. After fixation and permeabilization (BD) for 10 min, cells were stained intracellularly (see Table S1, <http://links.lww.com/QAD/A798>) and fixed in cellfix (BD) for flowcytometry.

Flow cytometry analysis

One hundred thousand events were acquired after phenotypical staining and 300 000 events were acquired after intracellular cytokine staining, using the LSRII flow cytometer (BD). Data were analysed using the DIVA software (BD). The events were gated for either lymphocytes or monocytes in a forward scatter-A versus sideward scatter plot. T-cell poly-functionality was analysed by Flowjo software (v9.2). Within the CD8⁺ T-cell population a Boolean gating was created for the five respective functions; CD107a, IFN γ , tumor necrosis factor alpha (TNF α), macrophage inflammatory protein (MIP)1 β , and interleukin (IL)-2, resulting in 31 different

combinations. All data were background-subtracted using the unstimulated samples. To convert the 5-dimensional poly-functional profile to a one-dimensional value taking into account all cells performing 0,1,2 until five functions we used the poly-functionality index, as developed and described previously [32].

Statistical analysis

Differences between groups were analysed using a Mann–Whitney test. All statistical analyses were performed using the software program SPSS 19.0 (SPSS Inc, Chicago, Illinois, USA). For further details, we refer to the legends of the figures.

Results

Study population

We included 36 HIV-1-infected individuals in our analyses. The viral declines of 16 of these individuals were published previously [26]. All patients were treated with at least a triple drug combination, and 35 of the individuals attained an undetectable viral load within 1 year. At baseline, viral loads ranged widely between individuals from 3340 to 5 220 000 HIV-1 RNA copies/ml, with a median of 72 200 HIV-1 RNA copies/ml, whereas CD4⁺ T-cell counts ranged from 30 to 851 cells/ μ l, with a median of 360 cells/ μ l (see Table 1). All, but one, of the study participants were male and the age of the participants varied from 25 to 60 years. The majority of individuals [24(67%)] initiated cART during chronic infection. Twenty-eight percent of the study population carried at least one of the protective HLA alleles HLA-B*27:05 or B*57:01, and 6% carried the detrimental HLA allele, namely HLA-B*35:03. The complete 2-field HLA-A and B typing of the study population is shown in Table S2, <http://links.lww.com/QAD/A798>.

Table 1. Baseline characteristics.

	Study population
Number	36
Age (years) ^a	40.1 (25.9–60.5)
Male ^b	35 (97%)
CD4 ⁺ T-cell count (x 10 ⁶ /l) ^a	360 (30–851)
HIV viral load (copies/ml) ^a	72 200 (3340–5 220 000)
Time since first positive HIV test (years) ^a	2.7 (0.1–10.4)
HLA background	
Protective HLA alleles (HLA B*27:05/B*57:01) ^b	10 (28%)
Detrimental HLA alleles (HLA B*35:03) ^b	2 (6%)
HLA homozygosity ^c	9 (27%)

^aMedian with range is given for total study population.
^bNumber and percentage is given for total study population.
^cHomozygosity at one or more of the HLA-A or B loci.

Large spread in the lifespans of productively infected cells

We investigated whether the lifespan of productively HIV-1-infected cells is shorter in individuals with CTL responses with features that are known to be associated with relatively slow HIV-1 disease progression. For each individual we estimated the lifespan of productively HIV-1-infected cells based on the slope of the viral decay after start of therapy, as published previously [27,28]. The rationale behind this approach is that effective cART prevents the formation of newly infected cells, such that the rate at which virus is lost on cART represents the rate at which productively HIV-infected cells were formed and lost prior to start of cART, when the viral load was still at steady state. For an accurate calculation of the first phase decline, at least four time points were measured in the first week after therapy initiation, as previously described [27,33,34]. Figure S2, <http://links.lww.com/QAD/A800> depicts the individual HIV-1 RNA declines

Fig. 1. Spread in lifespans of productively infected cells between individuals. An overview of the 36 individual HIV-1 RNA declines in the first week after start of antiretroviral therapy (a); the linear regression lines are depicted. The average lifespan of the productively infected cells of these individuals (b) was calculated as the inverse of the slopes of the regression lines of (a) (see Supplemental Figure 2, <http://links.lww.com/QAD/A800>, and Weverling *et al.* [26] for the individual viral RNA measurements). The results of the 16 individuals of the ERA-study are depicted in grey and of the 20 individuals of the THILIHT study in black.

of the newly included individuals. The viral decay of the other 16 study participants was published previously [26]. The expected lifespan of productively infected cells varied between individuals from 1.01 to 3.68 days (median 1.82 days, see Fig. 1 and Table S3, <http://links.lww.com/QAD/A798>). Despite their known association with the rate of progression to AIDS, neither baseline CD4⁺ T-cell count (Figure S3a, <http://links.lww.com/QAD/A801>) nor baseline plasma HIV-1 viral load (Figure S3b, <http://links.lww.com/QAD/A801>) correlated with the lifespan of productively infected cells [33].

The presence of protective HLA alleles does not affect the lifespan of productively infected cells

Because of the clear association between CD8⁺ T cells restricted through HLA-B27 and B57 and slow disease progression [5,8–11,35,36], we studied whether HLA genotypes associated with slow progression correlated with a reduced lifespan of productively infected cells. When the expected lifespans of productively infected cells in individuals with at least one of the protective HLA alleles HLA-B*27:05 or B*57:01 were compared with those who did not possess any protective HLA allele, we found no evidence that productively infected cells are shorter lived in people with a protective HLA molecule (Fig. 2a, $P=0.76$). Likewise, the relative hazard of the most protective HLA allele per individual did not correlate with the expected lifespan of productively infected cells (Fig. 2b, $r^2=0.004$, $P=0.72$). Productively infected cells were also not shorter-lived in individuals heterozygous for all HLA-A, -B and -C loci ($n=17$), another HLA phenotype related to delayed disease progression in HIV-1 infection [37] (Fig. 2c, $P=0.948$). Thus, the HLA background of an HIV-infected individual does not seem to affect the lifespan of productively infected cells.

No impact of Gag-specific CD8⁺ T cells on the lifespan of productively infected cells

As CD8⁺ T cells targeting Gag are associated with a relatively low viral load and slow HIV disease progression, even irrespective of their HLA restriction [8,10,15], we also investigated whether the magnitude, breadth and/or poly-functionality of Gag-specific CD8⁺ T-cell responses had an impact on the lifespan of productively infected cells. These experiments were performed in a subgroup of 20 participants in whom extra material was collected before start of cART.

The magnitude of the HIV-specific T-cell response against the overlapping peptide pool of Gag was measured using an IFN γ Elispot. As a control we also measured the responses elicited by the overlapping peptide pools of HIV Pol, Env and/or Nef. We found that the Gag-restricted T-cell response – when present – was dominant in all individuals. Despite its dominance, the magnitude of the CD8⁺ T-cell response against the Gag-peptide pool (Fig. 3a) was not associated with the lifespan

Fig. 2. No effect of the HLA background on the lifespan of productively infected cells. Panel (a) shows the lifespan of productively infected cells in individuals expressing one of the protective HLA alleles B*27:05 or B*57:01 ($n=10$) as compared with individuals without these HLA alleles ($n=26$). Panel (b) depicts the (lack of) correlation between the relative hazard of the most protective HLA A and HLA B allele of the 36 study participants and the lifespan of their productively infected cells. Panel (c) gives the lifespan of productively infected cells in individuals with a fully heterozygous ($n=27$) HLA background and in individuals with a partially homozygous ($n=9$) HLA background. Median lifespans in the different groups are depicted by horizontal lines. A linear regression is depicted in panel (b), with corresponding r^2 and P -values. The open dots/squares represent individuals with the detrimental HLA allele B*35:03.

Fig. 3. Characteristics of the HIV-specific CD8⁺ T-cell response are not associated with the lifespan of productively infected cells. In 20 HIV-infected individuals neither the magnitude (a), nor the breadth (b) or the functionality (c, d) of the Gag-specific CD8⁺ T-cell response was correlated with the lifespan of the productively infected cells. Linear regressions are depicted for each graph, with corresponding r^2 and P -values. Panel (c) shows that there were no significant differences in the expression of CD107a, IFN γ , IL-2, MIP1 β and TNF α by Gag-specific CD8⁺ T cells between the five individuals with the longest-lived productively infected cells (grey boxplots) and the five individuals with the shortest-lived productively infected cells (black boxplots). Median and range are depicted for each group.

of productively infected cells. Even when we compared individuals with the highest IFN γ response against HIV Gag (over 1000 SFU/10⁶ cells) with individuals without such a response, we observed no significant difference in the lifespan of their productively infected cells ($P = 0.74$, data not shown).

In addition, we studied the breadth of the Gag-specific T-cell response. We first made a 'quick scan' of the total Gag protein using an 11 by 11 matrixpool Elispot (see M&M), and combined with the use of a prediction programme this suggested that the total CTL response against Gag was driven by up to 15 potential epitopes per individual. For each individual, we subsequently confirmed which of these potential epitopes truly contributed to the immune response to Gag, by measuring whether the individual epitopes also elicited a T-cell response *in vitro*. This revealed a median breadth of 1.5

(range 0–7) Gag-specific CD8⁺ T-cell responses per individual. We observed no significant association between the number of CD8⁺ T-cell responses against HIV Gag and the lifespan of productively infected cells (Fig. 3b). Even in individuals with more than three responses against HIV Gag, which has previously been shown to be associated with a relatively low viral load [15], the productively infected cells did not live longer than in individuals with fewer than three responses against HIV Gag ($P = 0.65$, data not shown).

Finally, we determined the functional profile of the Gag-restricted CTL response in terms of the capacity to produce multiple cytokines/chemokines, including TNF α , MIP1 β , IFN γ , IL-2 and CD107a. A median of 1.5% of CD8⁺ T cells (range 0.2–4.5%) responded to HIV Gag (data not shown). We observed no significant differences between the specific functions exhibited by

the CD8⁺ T cells in response to HIV Gag of the five individuals with the shortest-lived and the five individuals with the longest-lived productively infected cells (Fig. 3c). In both groups, CD107a and MIP1 β production by HIV-specific CD8⁺ T cells made up approximately 80% of the total functional response whereas the contribution of TNF α and IFN γ was much lower, and IL-2 was hardly produced. When expressing the average number of functions of the Gag-specific T cells as a poly-functionality index [32], we did not find any association between T-cell poly-functionality and the lifespan of the productively infected cells (Fig. 3d). Even the four individuals with the most poly-functional T cells did not have significantly shorter-lived productively infected cells ($P=0.42$, data not shown). Also CD8⁺ T-cell exhaustion (as measured by the markers PD-1 and CD57) was not associated with the estimated lifespan of the productively infected cells (data not shown).

Discussion

Following the CD8⁺ T-cell depletion studies in rhesus-macaques [24,25], which showed no influence on the lifespan of productively SIV-infected cells, we studied whether shortening of the lifespan of productively infected cells may be limited to the most protective CTL responses only. Although the average lifespan of the productively infected human cells varied considerably between our study participants, it did not correlate with any of the factors known to be associated with slow disease progression, including the presence of protective HLA alleles, or a high, broad or poly-functional CD8⁺ T-cell response against HIV Gag. Thus, even the most protective HIV-specific CD8⁺ T-cell responses do not seem to exert their effect by shortening the lifespan of productively HIV-infected cells.

Although measuring the decay rate of plasma HIV-RNA following effective cART provides an elegant way to deduce the lifespan of productively infected cells before treatment, this approach may introduce a bias when applied to patients. In the past, cART was typically only initiated in asymptomatic persons because of certain clinical end-points, such as a decrease of the CD4⁺ T-cell count under a predefined value, or an increase in viral load. Under such circumstances, the HIV-specific CD8⁺ T cells may already have an exhausted phenotype, and therefore may not be representative for the typical HIV-specific T-cell response during chronic infection. Our study group is quite unique in this respect, because as many as 75% of our study participants started therapy *without* clinical progression. It is thus highly unlikely that the lack of effect of even the most protective HIV-specific CD8⁺ T-cell responses on the lifespan of productively infected cells that we report is due to such a bias. Indeed, we found no difference in the lifespan of productively

infected cells between individuals with acute or chronic HIV infection, and omitting the data from individuals who started therapy because of clinical progression did not change the conclusions of our study. It is important to note that we used the IFN γ -Elispot assay to measure CD8⁺ T-cell responses. This assay has been accepted as a general measure for CTL reactivity in many studies. Our conclusion that protective CD8⁺ T cells do not shorten the average lifespan of productively infected cells is not only based on the lack of correlation between IFN γ -producing HIV-specific CD8⁺ T cells and viral decay, but is also evident in the lack of correlation between the presence of protective HLA molecules and viral decay, reconfirming our conclusion.

In light of the plethora of prior evidence for a pivotal role of CD8⁺ T cells in HIV control [1,2,5,7,15,38], our findings suggest that CD8⁺ T-cell control of HIV infection is based on other mechanisms than cytolytic killing of productively infected cells. An alternative mechanism via which CD8⁺ T cells may reduce the viral load is through secretion of nonlytic soluble factors that interrupt HIV transcription [39], or reduce infection of uninfected cells. The best-known soluble noncytolytic factors secreted by CD8⁺ T cells are the CCR5-binding molecules RANTES, MIP1 α and MIP1 β . These chemokines function as competitive binders and down-modulators of the CCR5 receptor and thereby reduce HIV entry and infection [40]. Likewise, the cytokines IFN γ and TNF α are thought to have antiviral properties that can inhibit HIV-1 infection [41,42]. Indeed, depletion of CD8⁺ T cells in rhesus-macaques has been shown to lead to a marked reduction in the plasma levels of several antiviral cytokines and chemokines, including RANTES, MIP1 α , MIP1 β , IFN γ and TNF α [24,43]. An in-silico modelling study also demonstrated that nonlytic T cells can drive (slow) HIV immune escape, whereas previously it was presumed that HIV immune escape mutations were exclusively caused by lytic T cells [44]. In addition, in a study among SHIV-infected pigtail macaques it was shown that the dynamics of wildtype and escape mutant viral variants was compatible with a noncytolytic mechanism and not with cytolytic killing by CTL [45].

A second possibility is that CD8⁺ T cells perform their cytolytic effects *before* the productive stage of infection of the target-cells, namely within the first ~24 h after infection of the cell. Importantly, if target-cells can be recognized by CTL very early after they have been infected, they have not had the chance to produce new viral particles, and the expression of HLA molecules on the cell surface has not yet been down-regulated. Both our study and the two studies in rhesus-macaques would not detect such effects of CTL as the read-out was the death of productively infected cells. Indeed, it was shown that Gag-specific CD8⁺ T-cell clones are indeed able to recognize SIV-infected cells as early as 2 h after infection

of the target-cell [46]. The presented Gag epitopes are derived from the infecting virions, which can contain up to 5000 copies each [47]. Moreover, in HIV-1-infected humans a T-cell clone specific for the immunodominant HLA-B*27:05 Gag-restricted KK10 epitope recognized infected target-cells within 6 h postinfection, whereas sub-dominant HLA-B*27:05-restricted Vpr VL9 epitopes were not recognized until 18 h after infection [48]. Previous mathematical modelling has shown that such cytolytic effects of CTL on infected cells before their productive stage of infection could also reconcile the large inter-individual differences in set-point viral load, despite the relatively small inter-individual differences in the rate of viral-load decline during treatment [49]. It remains to be investigated which factors underlie these latter, albeit smaller, differences in viral dynamics on treatment.

It is fundamental to fully comprehend the mechanisms via which CD8⁺ T cells contribute to control of HIV infection. Puzzling outcomes of some vaccine trials have clearly shown how incomplete our understanding is of how CTL exert control. Despite a significant (although modest) positive outcome of the Thai vaccine trial, the absence of an effect on viral load and CD4⁺ T-cell counts, and extremely low CD8⁺ T-cell reactivity in the vaccinated, question the role of CTL to the induced protection [50]. Likewise, the STEP-trial had to be halted because of a lack of protection, no effect on viral set-points, and an increased risk of infection in vaccinated individuals with preexisting immunity against the viral vector [51]. Only if we fully understand the CD8⁺ T-cell-restricted mechanisms that control HIV-1 infection, will we be able to effectively apply this knowledge in the design of a functional HIV vaccine.

Acknowledgements

The Amsterdam Cohort Studies on HIV infection and AIDS, a collaboration between the Amsterdam Health Service, the Academic Medical Center of the University of Amsterdam, Sanquin Blood Supply Foundation and the University Medical Center Utrecht, are part of the Netherlands HIV Monitoring Foundation. Furthermore, we would like to thank Bart Grady for providing technical support.

Author contributions: H.B.S., I.M.M.S., M.N., T.M., D.v.B., and J.A.M.B. designed the study; H.B.S., T.F. and T.M., organized patient inclusion and sample collection; J.P., A.M.J.W., and E.S. provided data; H.B.S. performed the experiments; H.B.S., I.M.M.S., D.v.B., and J.A.M.B. analysed data; H.B.S., I.M.M.S., D.v.B., and J.A.M.B. wrote the manuscript. All authors were involved in critically appraising the manuscript and approve the final version.

Source of funding: This research has been funded by Aids Fonds Netherlands (grant 2010031 to JAMB).

Conflicts of interest

The authors have no conflicts of interest to disclose.

References

- Koup RA, Safrit JT, Cao Y, Andrews CA, McLeod G, Borkowsky W, *et al.* **Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome.** *J Virol* 1994; **68**:4650–4655.
- Schmitz JE, Kuroda MJ, Santra S, Sasseville VG, Simon MA, Lifton MA, *et al.* **Control of viremia in simian immunodeficiency virus infection by CD8⁺ lymphocytes.** *Science* 1999; **283**:857–860.
- Jin X, Bauer DE, Tuttleton SE, Lewin S, Gettie A, Blanchard J, *et al.* **Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques.** *J Exp Med* 1999; **189**:991–998.
- Matano T, Kobayashi M, Igarashi H, Takeda A, Nakamura H, Kano M, *et al.* **Cytotoxic T lymphocyte-based control of simian immunodeficiency virus replication in a preclinical AIDS vaccine trial.** *J Exp Med* 2004; **199**:1709–1718.
- Carrington M, O'Brien SJ. **The influence of HLA genotype on AIDS.** *Annu Rev Med* 2003; **54**:535–551.
- Fellay J, Shianna KV, Ge D, Colombo S, Ledergerber B, Weale M, *et al.* **A whole-genome association study of major determinants for host control of HIV-1.** *Science* 2007; **317**:944–947.
- Pereyra F, Jia X, McLaren PJ, Telenti A, de Bakker PI, Walker BD, *et al.* **The major genetic determinants of HIV-1 control affect HLA class I peptide presentation.** *Science* 2010; **330**:1551–1557.
- Almeida JR, Price DA, Papagno L, Arkoub ZA, Sauce D, Bornstein E, *et al.* **Superior control of HIV-1 replication by CD8⁺ T cells is reflected by their avidity, polyfunctionality, and clonal turnover.** *J Exp Med* 2007; **204**:2473–2485.
- Almeida JR, Sauce D, Price DA, Papagno L, Shin SY, Moris A, *et al.* **Antigen sensitivity is a major determinant of CD8⁺ T-cell polyfunctionality and HIV-suppressive activity.** *Blood* 2009; **113**:6351–6360.
- Betts MR, Nason MC, West SM, De Rosa SC, Migueles SA, Abraham J, *et al.* **HIV nonprogressors preferentially maintain highly functional HIV-specific CD8⁺ T cells.** *Blood* 2006; **107**:4781–4789.
- Migueles SA, Laborico AC, Shupert WL, Sabbaghian MS, Rabin R, Hallahan CW, *et al.* **HIV-specific CD8⁺ T cell proliferation is coupled to perforin expression and is maintained in non-progressors.** *Nat Immunol* 2002; **3**:1061–1068.
- Bussey F, Le CJ, Corre B, Porrot F, Burgard M, Rouzioux C, *et al.* **Inverse correlation between memory Gag-specific cytotoxic T lymphocytes and viral replication in human immunodeficiency virus-infected children.** *J Infect Dis* 2002; **186**:1589–1596.
- Edwards BH, Bansal A, Sabbaj S, Bakari J, Mulligan MJ, Goepfert PA. **Magnitude of functional CD8⁺ T-cell responses to the Gag protein of human immunodeficiency virus type 1 correlates inversely with viral load in plasma.** *J Virol* 2002; **76**:2298–2305.
- Honeyborne I, Prendergast A, Pereyra F, Leslie A, Crawford H, Payne R, *et al.* **Control of human immunodeficiency virus type 1 is associated with HLA-B*13 and targeting of multiple Gag-specific CD8⁺ T-cell epitopes.** *J Virol* 2007; **81**:3667–3672.
- Kiepiela P, Ngumbela K, Thobakgale C, Ramduth D, Honeyborne I, Moodley E, *et al.* **CD8⁺ T-cell responses to different HIV proteins have discordant associations with viral load.** *Nat Med* 2007; **13**:46–53.
- Masemola A, Mashishi T, Khoury G, Mohube P, Mokgotho P, Vardas E, *et al.* **Hierarchical targeting of subtype C human immunodeficiency virus type 1 proteins by CD8⁺ T cells: correlation with viral load.** *J Virol* 2004; **78**:3233–3243.
- Novitsky V, Gilbert P, Peter T, McLane MF, Gaolekwe S, Rybak N, *et al.* **Association between virus-specific T-cell responses and plasma viral load in human immunodeficiency virus type 1 subtype C infection.** *J Virol* 2003; **77**:882–890.

18. Zuniga R, Lucchetti A, Galvan P, Sanchez S, Sanchez C, Hernandez A, *et al.* **Relative dominance of Gag p24-specific cytotoxic T lymphocytes is associated with human immunodeficiency virus control.** *J Virol* 2006; **80**:3122–3125.
19. Borghans JA, Molgaard A, de Boer RJ, Kesmir C. **HLA alleles associated with slow progression to AIDS truly prefer to present HIV-1 p24.** *PLoS One* 2007; **2**:e920.
20. Brockman MA, Schneidewind A, Lahaie M, Schmidt A, Miura T, Desouza I, *et al.* **Escape and compensation from early HLA-B57-mediated cytotoxic T-lymphocyte pressure on human immunodeficiency virus type 1 Gag alter capsid interactions with cyclophilin A.** *J Virol* 2007; **81**:12608–12618.
21. Schneidewind A, Brockman MA, Yang R, Adam RI, Li B, Le GS, *et al.* **Escape from the dominant HLA-B27-restricted cytotoxic T-lymphocyte response in Gag is associated with a dramatic reduction in human immunodeficiency virus type 1 replication.** *J Virol* 2007; **81**:12382–12393.
22. Schneidewind A, Brockman MA, Sidney J, Wang YE, Chen H, Suscovich TJ, *et al.* **Structural and functional constraints limit options for cytotoxic T-lymphocyte escape in the immunodominant HLA-B27-restricted epitope in human immunodeficiency virus type 1 capsid.** *J Virol* 2008; **82**:5594–5605.
23. Davenport MP, Petravic J. **CD8+ T cell control of HIV—a known unknown.** *PLoS Pathog* 2010; **6**:e1000728.
24. Klatt NR, Shudo E, Ortiz AM, Engram JC, Paiardini M, Lawson B, *et al.* **CD8+ lymphocytes control viral replication in SIV-mac239-infected rhesus macaques without decreasing the lifespan of productively infected cells.** *PLoS Pathog* 2010; **6**:e1000747.
25. Wong JK, Strain MC, Porra R, Reay E, Sankaran-Walters S, Ignacio CC, *et al.* **In vivo CD8+ T-cell suppression of SIV viremia is not mediated by CTL clearance of productively infected cells.** *PLoS Pathog* 2010; **6**:e1000748.
26. Weverling GJ, Lange JM, Jurriaans S, Prins JM, Lukashov VV, Notermans DW, *et al.* **Alternative multidrug regimen provides improved suppression of HIV-1 replication over triple therapy.** *AIDS* 1998; **12**:F117–F122.
27. Ho DD, Neumann AU, Perelson AS, Chen W, Leonard JM, Markowitz M. **Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection.** *Nature* 1995; **373**:123–126.
28. Wei X, Ghosh SK, Taylor ME, Johnson VA, Emini EA, Deutsch P, *et al.* **Viral dynamics in human immunodeficiency virus type 1 infection.** *Nature* 1995; **373**:117–122.
29. Boyum A. **Isolation of mononuclear cells and granulocytes from human blood. Isolation of monuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g.** *Scand J Clin Lab Invest Suppl* 1968; **97**:77–89.
30. Schellens IM, Kesmir C, Miedema F, Van BD, Borghans JA. **An unanticipated lack of consensus cytotoxic T lymphocyte epitopes in HIV-1 databases: the contribution of prediction programs.** *AIDS* 2008; **22**:33–37.
31. Mashishi T, Gray CM. **The ELISPOT assay: an easily transferable method for measuring cellular responses and identifying T cell epitopes.** *Clin Chem Lab Med* 2002; **40**:903–910.
32. Larsen M, Sauce D, Arnaud L, Fastenackels S, Appay V, Gorochov G. **Evaluating cellular polyfunctionality with a novel polyfunctionality index.** *PLoS One* 2012; **7**:e42403.
33. Klenerman P, Phillips RE, Rinaldo CR, Wahl LM, Ogg G, May RM, *et al.* **Cytotoxic T lymphocytes and viral turnover in HIV type 1 infection.** *Proc Natl Acad Sci USA* 1996; **93**:15323–15328.
34. Perelson AS, Neumann AU, Markowitz M, Leonard JM, Ho DD. **HIV-1 dynamics in vivo: virion clearance rate, infected cell lifespan, and viral generation time.** *Science* 1996; **271**:1582–1586.
35. Gao X, Nelson GW, Karacki P, Martin MP, Phair J, Kaslow R, *et al.* **Effect of a single amino acid change in MHC class I molecules on the rate of progression to AIDS.** *N Engl J Med* 2001; **344**:1668–1675.
36. Gao X, Bashirova A, Iversen AK, Phair J, Goedert JJ, Buchbinder S, *et al.* **AIDS restriction HLA allotypes target distinct intervals of HIV-1 pathogenesis.** *Nat Med* 2005; **11**:1290–1292.
37. Carrington M, Nelson GW, Martin MP, Kissner T, Vlahov D, Goedert JJ, *et al.* **HLA and HIV-1: heterozygote advantage and B*35-Cw*04 disadvantage.** *Science* 1999; **283**:1748–1752.
38. Moore CB, John M, James IR, Christiansen FT, Witt CS, Mallal SA. **Evidence of HIV-1 adaptation to HLA-restricted immune responses at a population level.** *Science* 2002; **296**:1439–1443.
39. Mackewicz CE, Blackburn DJ, Levy JA. **CD8+ T cells suppress human immunodeficiency virus replication by inhibiting viral transcription.** *Proc Natl Acad Sci USA* 1995; **92**:2308–2312.
40. Cocchi F, DeVico AL, Garzino-Demo A, Arya SK, Gallo RC, Lusso P. **Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells.** *Science* 1995; **270**:1811–1815.
41. Boehm U, Klamp T, Groot M, Howard JC. **Cellular responses to interferon-gamma.** *Annu Rev Immunol* 1997; **15**:749–795.
42. Herbein G, Montaner LJ, Gordon S. **Tumor necrosis factor alpha inhibits entry of human immunodeficiency virus type 1 into primary human macrophages: a selective role for the 75-kilodalton receptor.** *J Virol* 1996; **70**:7388–7397.
43. Bosinger SE, Jochems SP, Folkner KA, Hayes TL, Klatt NR, Silvestri G. **Transcriptional profiling of experimental CD8(+) lymphocyte depletion in rhesus macaques infected with simian immunodeficiency virus SIVmac239.** *J Virol* 2013; **87**:433–443.
44. Seich AI, Basatena NK, Chatzimichalis K, Graw F, Frost SD, Regoes RR, Asquith B. **Can nonlytic CD8+ T cells drive HIV-1 escape?** *PLoS Pathog* 2013; **9**:e1003656.
45. Balamurali M, Petravic J, Loh L, Alcantara S, Kent SJ, Davenport MP. **Does cytotoxicity by CD8+ T cells drive immune escape in HIV infection?** *J Immunol* 2010; **185**:5093–5101.
46. Sacha JB, Chung C, Rakasz EG, Spencer SP, Jonas AK, Bean AT, *et al.* **Gag-specific CD8+ T lymphocytes recognize infected cells before AIDS-virus integration and viral protein expression.** *J Immunol* 2007; **178**:2746–2754.
47. Briggs JA, Simon MN, Gross I, Krausslich HG, Fuller SD, Vogt VM, *et al.* **The stoichiometry of Gag protein in HIV-1.** *Nat Struct Mol Biol* 2004; **11**:672–675.
48. Payne RP, Kloverpris H, Sacha JB, Brumme Z, Brumme C, Buus S, *et al.* **Efficacious early antiviral activity of HIV Gag- and Pol-specific HLA-B 2705-restricted CD8+ T cells.** *J Virol* 2010; **84**:10543–10557.
49. Althaus CL, de Boer RJ. **Implications of CTL-mediated killing of HIV-infected cells during the nonproductive stage of infection.** *PLoS One* 2011; **6**:e16468.
50. Rerks-Ngarm S, Pitisuttithum P, Nitayaphan S, Kaewkungwal J, Chiu J, Paris R, *et al.* **Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand.** *N Engl J Med* 2009; **361**:2209–2220.
51. Buchbinder SP, Mehrotra DV, Duerr A, Fitzgerald DW, Mogg R, Li D, *et al.* **Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial.** *Lancet* 2008; **372**:1881–1893.