Published in final edited form as:

AIDS. 2015 January 2; 29(1): 23–33. doi:10.1097/QAD.0000000000000508.

Broad and persistent Gag-specific CD8+ T-cell responses are associated with viral control but rarely drive viral escape during primary HIV-1 infection

Mopo Radebe^{a,b}, Kamini Gounder^{a,b}, Mammekwa Mokgoro^a, Zaza M. Ndhlovu^{a,c}, Zenele Mncube^a, Lungile Mkhize^a, Mary van der Stok^a, Manjeetha Jaggernath^a, Bruce D. Walker^{a,b,c,d}, and Thumbi Ndung'u^{a,b,c,e}

Mopo Radebe: Leshwedi@ukzn.ac.za; Kamini Gounder: GounderK@ukzn.ac.za; Mammekwa Mokgoro: Mokgoro@ukzn.ac.za; Zaza M. Ndhlovu: zndhlovu@mgh.harvard.edu; Zenele Mncube: Mncube@ukzn.ac.za; Lungile Mkhize: Mkhizel2@ukzn.ac.za; Mary van der Stok: Vanderstok@ukzn.ac.za; Manjeetha Jaggernath: Jaggernath@ukzn.ac.za; Bruce D. Walker: bwalker@mgh.harvard.edu; Thumbi Ndung'u: ndungu@ukzn.ac.za

^aHIV Pathogenesis Programme, Doris Duke Medical Research Institute, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, 719 Umbilo Road, Durban, South Africa

^bKwaZulu-Natal Research Institute for Tuberculosis and HIV, Nelson R. Mandela School of Medicine, 719 Umbilo Road, Durban, South Africa

^cRagon Institute of Massachusetts General Hospital, Massachusetts Institute of Technology and Harvard University, 400 Technology Square, Cambridge, Massachusetts, United States of America

^dHoward Hughes Medical Institute, 4000 Jones Bridge Road Chevy Chase, Maryland, United States of America

^eMax Planck Institute for Infection Biology, Charitestraße 1, Berlin, Germany

Abstract

Objective—We characterized protein-specific CD8+ T-cell immunodominance patterns during the first year of HIV-1 infection, and their impact on viral evolution and immune control.

Methods—We analyzed CD8+ T-cell responses to the full HIV-1 proteome during the first year of infection in eighteen antiretroviral-naïve individuals with acute HIV-1 subtype C infection, all identified prior to seroconversion. *Ex vivo* and cultured IFN- γ ELISPOT assays were performed and viruses from plasma were sequenced within defined CTL Gag epitopes.

Results—Nef-specific CD8+ T-cell responses were dominant during the first 4 weeks post infection and made up 40% of total responses at this time, yet by 1 year responses against this region had declined and Gag responses made up to 47% of all T-cell responses measured. An inverse correlation between the breadth of Gag-specific responses and viral load set point was evident at 26 weeks post infection ($p=0.0081$; $r=-0.60$) and beyond. An inverse correlation between the number of persistent responses targeting Gag and viral set point was also identified ($p=0.01$; $r=-0.58$). Gag-specific responses detectable by the cultured ELISPOT assay correlated

negatively with viral load set point ($p=0.0013$; $r=-0.91$). Sequence evolution in targeted and non-targeted Gag epitopes in this cohort was infrequent.

Conclusions—These data underscore the importance of HIV-specific CD8+ T-cell responses, particularly to the Gag protein, in the maintenance of low viral load levels during primary infection and show that these responses are initially poorly elicited by natural infection. These data have implications for vaccine design strategies.

Keywords

CD8+ T-cells; Gag-specific responses; Acute HIV-1 infection; Persistent CD8+ T-cell responses; Central CD8+ T-cell memory responses; Immune escape

Introduction

There is compelling evidence that acute-phase HIV-specific CD8+ T-cells play an important role in determining the clinical course of disease. Experimental data and mathematical models show that the appearance of HIV-1-specific CD8+ T-cells coincides with and contributes to the decline in peak viremia in acute infection [1–5], and in some cases virus-specific CD8+ T-cells precede antibodies [3, 5–7]. An indicator of CD8+ T-cell immune pressure on the virus is the emergence of viral escape within CTL epitopes, which can occur as early as 10 days after detection of a CD8+ T-cell response following HIV-1 infection [3, 8].

Despite strong data that HIV-1-specific CD8+ T-cells play a critical role in viral control, there is also evidence of marked heterogeneity in their antiviral effectiveness. First, is the observation that almost all HIV-1-infected individuals have detectable T-cell responses, irrespective of viral control [9, 10], suggesting inefficient viral control by some CD8+ T-cells [11–16]. Secondly, it has been demonstrated that CD8+ T-cells targeting different viral proteins differ in their antiviral effectiveness [12], consistent with clinical data that Gag but not Env- or Nef-specific responses are associated with lower viremia [12, 17–21]. Third, there is variability in the hierarchy of responses following infection and the ability of HLA-restricted CD8+ T-cell responses to drive viral immune escape is not consistent and remains incompletely characterized [5, 22, 23].

Studies of early HIV-1 infection offer an opportunity to unravel properties underlying CD8+ T-cell effectiveness. Although numerous studies have examined CD8+ T-cells in early infection [1, 2, 8, 11, 21, 23–28], less is known about the impact and fate these responses in persons with clearly defined dates of infection in whom studies have been initiated within a month of exposure to the virus [3, 7, 11, 13, 21, 24, 29]. A number of studies have reported on the longitudinal analysis of CD8+ T-cell responses in relation to time from initial acute or primary infection and viral load, in order to determine the kinetics and fate of these responses and their impact on the virus over time [3, 5, 13, 19, 21–24, 30]. However, few have assessed CD8+ T-cells to the entire viral proteome during acute infection and at one year in antiretroviral-naïve persons, in high prevalence and incidence settings [5, 24]. This is an important consideration in view of evidence of viral adaptation to endemic population

HLA and loss of CTL epitopes over the course of the epidemic [31, 32] and the emerging evidence of high transmission of escape variants [33, 34].

Here, using a cohort of acutely infected individuals in a high HIV-1 prevalence setting, we defined the specificity of the earliest CD8+ T-cell responses, longitudinally characterized the fate of these responses and their impact on viral control within the first year of infection. Our data provide evidence of marked differences in immunogenicity of individual viral proteins for induction of HIV-specific CD8+ T-cell responses during acute HIV infection, and lack of a clear relationship of early CD8+ T-cell responses to viral load. However, over a one year period of follow up, the number of targeted Gag CD8+ T-cell epitopes increased resulting in a significant negative association between these responses and viral load set point. This information sheds light on the characteristics of evolving CD8+ T-cell responses, which mediate long-term control of HIV-1 infection in a high incidence setting.

Methods

Study participants

The cohort consisted of 18 antiretroviral-naïve individuals with acute HIV-1C infection identified in Durban, South Africa [7]. Briefly, study participants were recruited from HIV Counseling and Testing centers based on the criteria of being HIV rapid immunoassay negative and plasma RNA positive. Blood samples were obtained from study participants at 2, 4, 6, 8, 12, 18, 26 and 52 weeks post-infection. The date of infection was estimated to be 14 days prior to screening, as previously described [7]. The median age at recruitment was 28 years (interquartile range [IQR] 26 to 40 years), and 60% of the participants were female. The median plasma viral load at enrollment was 5,572,160 RNA copies/ml, (IQR, 921,090 to 9,320,000 RNA copies/ml) and the median CD4 count was 389 cells/mm³ (IQR, 275 to 496 cells/mm³). The average viral load between 3 and 12 months post infection was the measure of viral load set point used in this study. The Biomedical Research Ethics Committee of the University of KwaZulu-Natal approved this study, and participants provided written informed consent.

Synthetic HIV-1 subtype C peptides

A panel of 410 consensus subtype C peptides (18-mers overlapping by 10 amino acid residues), spanning the entire HIV-1 proteome were synthesized on an automated peptide synthesizer (MBS 396; Advanced ChemTech, Louisville, Kentucky) and used in the ELISPOT assay as previously described [35].

Viral load determination, CD4 T-cell counts, and HLA typing

Plasma HIV-1 RNA levels were quantified using the Roche Amplicor version 1.5 or Cobas Taqman HIV-1 Test according to the manufacturer's instructions (Roche Diagnostics, Branchburg, NJ). Absolute blood CD4+ T-cell counts were enumerated using Tru-Count technology on a FACSCalibur flow cytometer (Becton Dickinson). High-resolution HLA typing was performed as previously described [7].

Interferon- γ ELISPOT Assay

IFN- γ enzyme-linked immunosorbent spot assays (ELISPOT) were performed as described previously [7, 35, 36]. Briefly, peripheral blood mononuclear cell (PBMC) samples were stimulated with HIV-1 peptide pools (2 μ g/ml) and incubated overnight at 37°C with 5% CO₂. Phytohemagglutinin was used as a positive control and negative controls consisted of cells without stimuli. Confirmation of positive responses at the single-peptide level within peptide pools was undertaken in a second ELISPOT assay. A response was defined as positive when there were at least 100 spot-forming cells (SFCs)/million PBMCs and the total number of spots was 3 standard deviations above the negative control value [37, 38]. HLA class I-restricted epitopes predicted from the published epitopes on the HIV immunology database [39], based on the expressed HLA alleles of the participants were also tested.

Cultured IFN- γ ELISPOT Assay

The assays were performed as described previously [40, 41]. Briefly, 5–10 $\times 10^6$ peptide-stimulated PBMCs were cultured at 37°C and 5% CO₂ for 12 days in 5–10 ml of RPMI medium containing 10% heat-inactivated fetal calf serum (R10 medium) and supplemented with 50 units/ml of recombinant human IL-2 (R10/50 medium). Unstimulated cells cultured with IL-2 (R10/50 medium) alone were used as controls. A peptide concentration of 100 ng/ml (the final concentration of each peptide within the pool) was selected for these experiments. Fresh R10/50 medium was added to the cultures at days 3, 7, and 10 as needed. On day 12, cells were harvested, washed three times with fresh R10 medium and rested at 37°C and 5% CO₂ overnight in fresh R10 medium. Cells were then plated for a standard ELISPOT assay as described for the *ex vivo* ELISPOT.

Virus sequencing

Viral RNA was isolated plasma samples using the QIAamp Viral RNA Extraction Mini Kit (Qiagen, Hilden, Germany). Viral RNA was then reverse transcribed using ThermoScript™ RT-PCR System kit (Invitrogen, Carlsbad, CA, USA) and the gene-specific primer, GagD reverse as previously described [42]. Sequencing was done using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit version 3.4 (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Statistical analysis and graphical presentation were performed using GraphPad Prism version 5.0 software. Pearson's or Spearman's correlation was used to assess the relationship between immune responses and viral load. Statistical analysis of significance was calculated using Friedman ANOVA with Dunn's test for multiple comparisons. A value of $p < 0.05$ was considered statistically significant.

Results

Dynamics of HIV-1-specific T-cell responses and association with viral load

In earlier studies, we examined the specificity of CD8⁺ T-cell responses to HIV during the first 18 weeks of infection in twenty subjects with acute HIV infection [7]. Here we extended these studies by examining changes in the frequency of recognition, magnitude and breadth of HIV-1-specific CD8⁺ T-cell responses during the entire first year of infection.

We screened 18 subjects using the IFN- γ ELISPOT assay with synthetic peptides spanning HIV-1 Gag, Pol, Env, Nef, Tat, Vpr, Rev, Vif, and Vpu as described under materials and methods. A summary of the defined class I MHC-restricted HIV-1 epitopes targeted per subject is depicted in Figure 1.

At 4 weeks post infection, the earliest time point of measurement of HIV-specific CD8⁺ T-cell responses, 55% of the subjects made Nef-specific T-cell responses, followed by Gag (33%), Pol (27%), VVTRV (Vif, Vpr, Vpu, Rev and Tat) (16%) and Env (10%) (Figure 1A). Responses against Nef increased in frequency and between 12 and 18 weeks, 77% of the subjects had detectable Nef responses, subsequently, these responses underwent a marked decline and at 52 weeks only 55% of the subjects had detectable anti-Nef responses. In contrast, epitopes in the Gag region were targeted by only 33% of the subjects at 4 weeks and this number increased to 83% at 52 weeks. The number of individuals with detectable responses against Pol peaked at 12 weeks when 94% of subjects had detectable anti-Pol responses.

Of the responses measured against both overlapping peptides (OLPs) and optimal epitopes (8–11mer), 88% were confirmed to be CD8⁺ T-cell mediated based on known HLA restriction or recognition of 9–11 amino acid epitopes. However, 9% of OLP responses were detected in the absence of responses against defined class I MHC-restricted HIV-1-specific CD8⁺ epitopes known to be contained within these OLPs and 8 (3%) were against OLPs that have not been defined, raising the possibility that these may be either CD4⁺ or novel CD8⁺ T-cell responses. The sequences of the defined class I MHC-restricted HIV-1-specific CD8⁺ epitopes that were targeted, the restricting MHC class I allele, and position of these epitopes in the HIV-1 genome as well as OLPs that were targeted but have not been previously defined are listed in supplementary Tables 1 and 2.

In those in whom HIV-1-specific T-cell responses were detected, the mean magnitude of responses was variable over time (Figure 1B). In comparison to responses directed against other regions at 4 weeks, the mean magnitude of responses targeting epitopes in Nef were higher in magnitude followed by Env, 1,100 SFC/million PBMC and 720 SFC/million PBMC, respectively (Figure 1B). In contrast, the mean magnitude of Gag responses were the lowest at 320 SFC/million PBMC at this time and although fluctuations were observed in the magnitude of responses targeting other regions during the one year of infection, the mean maximal magnitude of Gag responses was reached at 8 weeks and was maintained up to 52 weeks post infection.

Evolution of immune responses was also apparent in terms of the breadth of responses to individual viral proteins. For this analysis we examined the sum total responses detected in the 18 study subjects. A total of 41 T-cell responses against epitopes in Gag, Pol, Nef, Env and VVTRV were measured at 4 weeks. As expected, Nef responses were dominant in breadth (Figure 1C) and made 42% of the total detectable HIV-specific CD8+ T-cell response at this time, whilst Pol and Gag-specific responses contributed 24% and 17%, respectively. Yet with increased duration of infection there was a steady rise in the overall number of Gag epitopes targeted (Figure 1C–H) and the highest breadth of Gag-specific responses was recorded at 52 weeks post-infection, the last time point to be analyzed (Figure 1F). At the last time point to be analyzed, Gag responses were most dominant in breadth (Figure 1F) and made up to 47% of the total detectable HIV-specific CD8+ T-cell response at this time, whilst Pol and Nef-specific responses contributed 19% and 13%, respectively.

We next aimed to study the impact of magnitude or breadth of CD8+ T-cell responses disease progression in the first 12 months post infection. We observed no correlation between magnitude and breadth of all of responses to epitopes in Pol, Env, Rev, Tat, Vpu, Vpr and Vif and viral load set point at any time point analyzed (data not shown). There was also no significant association between the breadth of Nef T-cell responses and viral set point at any time point, albeit for a trend towards positive correlation noted at 8 weeks ($p=0.098$; $r=0.4$) (Figure 2A). Although there was no correlation observed between the breadth of Gag-specific responses measured up to 18 weeks and viral set point (Figure 2B and data not shown), subsequent measures showed a significant negative association from 26 weeks ($p=0.0081$; $r=-0.61$) and at 52 weeks ($p=0.017$; $r=-0.57$) (Figure 2B–D). When a linear regression model was fitted, accounting for the repeated measurements and adjusting for weeks post infection, there remained a significant association between breadth and viral load set point, such that for every 1 unit increase in breadth, viral load decreases by 0.15 log copies/ml (SE=0.067; $p=0.0225$; data not shown).

The fate of the early T-cell responses and their impact on viremia

As illustrated in Figure 1, over the first year of infection, CD8+ T-cell responses are highly variable. Even at an epitope-specific level within each viral protein, immune responses fluctuated with some persisting throughout, some no longer detectable at later time points while others were undetectable at some time points and later re-emerged [7]. Few studies have examined the relationship between early loss and gain in CD8+ T-cell responses and viral load [3, 24, 43] and this led us to investigate the fate of these early responses and their impact on viral load set point. We focused our analysis on two distinct recognition profiles, persistent and lost responses. Persistent responses were defined as responses that were detected as early as 6 weeks and persisted up to 52 weeks. Lost responses were defined as responses which appeared at any time during the follow up period but had decreased by more than 80% and/or had fallen below the threshold of 100 SFC/ 10^6 PBMC at 52 weeks post infection.

Analysis revealed that of 92 HIV-1-specific CD8+ T-cell responses detected at 6 weeks, only 41 (45%) persisted at 52 weeks. Of the 16 Gag-specific responses measured at 6 weeks, 12 (75%) persisted and were still detectable at 52 weeks. Table 1 shows a summary of

persistent responses. All subjects had persisting T-cell responses against either optimal epitopes (36 in total) and/or OLPs (5 in total), ranging from 1 to 5 responses per subject. Responses to Pol made up 34% of all persistent responses, followed by Gag (29%), and Nef (15%). When T-cell responses were analysed irrespective of viral protein or region targeted, there was no significant association between the breadth of responses and viral load set point ($P=0.37$; $r=-0.22$) (Figure 3A). Even though Pol-specific responses made up 34% of all persistent responses, there was no correlation between breadth of these responses and viral load set point ($P=0.77$; $r=-0.073$) (Figure 3B). In contrast, a significant association was observed between Gag persistent responses and viral set point ($P=0.010$; $r=-0.59$) (Figure 3C), suggesting that persistent Gag-specific CD8+ responses may be advantageous in providing a sustained reduction in viremia.

Whilst some of the early HIV-1-specific IFN- γ ELISPOT CD8+ T-cell responses persisted, 55% of responses waned and were below the detection threshold at 52 weeks. To determine the fate of some of these responses which were no longer detectable, we used the cultured IFN- γ ELISPOT assay which assesses the proliferation of low frequency T-cells [40, 41, 44]. Sample availability limited evaluation by the cultured ELISPOT assay to only 8 subjects. In these 8 individuals, a total of 96 (median=11) *ex vivo* ELISPOT responses were measured between weeks 4 and 26 post infection (Supplementary Table 3). These responses fluctuated over time and only 30% were still detectable by the *ex vivo* ELISPOT assay at 52 weeks. Of the 67 individual epitope-specific T-cell responses which had disappeared and were presumed lost, 27 (40%) of these responses could be detected with the cultured ELISPOT assay. These responses were scattered across the proteome, with 30% of responses targeting Gag epitopes. There was no significant correlation between the presence of cultured ELISPOT responses targeting all HIV-1 regions and viral set point ($P=0.24$; $r=0.47$ and data not shown). However, a significant negative correlation between cultured ELISPOT Gag-specific responses and viral set point ($P=0.017$; $r=-0.85$) (Figure 3D) was observed. In summary, the cultured ELISPOT amplified and enhanced the detection of at least 40% of low frequency T-cell responses, and low frequency Gag-specific responses appear to mediate viral control.

Gag immune responses and viral evolution in primary HIV-1 infection

To investigate the relationship between Gag-specific T-cell responses and sequence evolution within targeted Gag epitopes, we sequenced HIV-1 *gag* from plasma samples obtained at 2–6 weeks and 52 weeks post infection. We evaluated a cumulative total of 210 epitopes restricted by the HLA class I alleles expressed by the study participants. Of the epitope sequences assessed at 2–6 weeks, T-cell responses were only mounted against 14% (11 of 78) of the wild type epitopes known to be presented in the context of HLA alleles expressed by the subject (Figure 4A). Interestingly, 7% (9 of 135) of epitopes with variant sequences either in the putative epitope or in the flanking region also induced a detectable T-cell response. Analysis at 52 weeks showed that a significantly higher number of both wild type (24 of 75, 32%) ($P=0.006$) and variant epitopes (21 of 135, 15%) ($P=0.01$) respectively induced an *ex vivo* ELISPOT detectable CD8+ T-cell response. A total of 11 Gag-specific T-cell responses against wild type epitopes were measured at 2–6 weeks but mutations had occurred in only two of these epitopes, the HLA-B*57-TW10 and B*81-TL9 by one year,

and yet these two epitopes still induced a detectable response at this time (Figure 4B). In contrast, CD8⁺ T-cell responses against HLA-B*8-EV9 and HLA-B*15:10-VL10 were no longer detectable after 6 weeks and these wild type epitope sequences failed to induce a detectable response even up to 52 weeks. Responses targeting 6 of the epitopes with variant sequences at 2–6 weeks declined over time yet this loss of response did not coincide with further sequence changes within the epitopes or their flanking regions (Figure 4C). There was no significant difference in viral set point between participants who made early T-cell responses to wild type epitopes and retained these responses even when variant sequences arose (median=4.1 log₁₀ copies/ml) and participants who made early T-cell responses against variant epitope sequences and retained these responses (median=4.4 log₁₀ copies/ml) ($p=0.416$). However, the former had a significantly lower viral load set point values in comparison to participants who made responses to epitopes with variant sequences but subsequently lost these responses (median=5.2 log₁₀ copies/ml) ($p=0.003$).

Discussion

Data over the past years have established that early immune events determine the rate of HIV/AIDS disease progression and thus a detailed understanding of properties of effective CD8⁺ T-cells in early infection may inform vaccine and therapeutic strategies [26, 27, 45]. In the current study we have analyzed the evolution of HIV-specific T-cell responses during the first year of infection, investigated their association with viral load set point, their ability to persist and their ability to induce viral escape. A diverse pattern of T-cell recognition across the HIV-1 proteome was evident during primary infection, with significant heterogeneity among participants in the timing of appearance and dominance of virus-specific T-cell responses. Although Nef responses were initially dominant in magnitude and breadth, Gag-specific responses steadily increased and had become dominant by one year post infection. A significant finding in the current study is the inverse association between the viral load set point and breadth of Gag responses measured during early infection but not those measured at the most acute phase of infection. Our findings provide a clear demonstration of the broadening of Gag-specific T-cell responses over the course of one year following HIV-1 infection, with this increased immunogenicity associated with lower viral load set point. Our findings are in line with other studies, which have shown that the presence of a high proportion of Gag-specific CD8⁺ T-cell responses correlates with delayed disease progression [19–21, 23, 46–48].

The paucity of Gag-specific responses during the acute stages of HIV-1 infection when dramatic declines in viremia are recorded suggest that Gag-specific T-cell responses may be more important in maintaining rather than determining the viral load set point [3, 43, 49]. Importantly, and relevant for vaccine design, our results imply that relatively limited Gag immunogenicity during acute HIV-1 infection leads to suboptimal viral control. Thus, a vaccine designed to induce greater breadth of Gag-specific CD8⁺ T-cell responses may tilt the balance towards control during the earliest phases of infection and result in more efficient subsequent viral control than would be expected in natural infection.

We also show that individuals with controlled viremia had significantly higher Gag-specific T-cell responses, which persisted from the first 6 weeks up to 52 weeks post infection. These

findings confirm that fluctuations in levels of T-cell responses and viral load are related even in early HIV-1 infection. The fluctuations of HIV-specific CD8+ T-cell responses during primary infection led us to investigate the impact of these changes on disease progression. Consistent with previous studies, some antigen-specific CD8+ T-cell responses undetectable by the *ex vivo* ELISPOT assay could be detected by the cultured ELISPOT assay, suggesting that they were maintained as low frequency central memory precursors [40, 41]. However, it cannot be ruled out that these responses represent low frequency CD8+ T-cells that are functionally and phenotypically indistinguishable from those detected by the regular ELISPOT assay [50]. We noted a significant negative correlation between the presence of cultured ELISPOT Gag-specific T-cell responses and viral set point, consistent with previous studies showing antiviral activity of these cells [40] and that preservation of memory T-cells is essential for better outcome and survival in HIV-1 infection [41, 51–54].

HIV-1 Gag sequencing revealed that despite host expression of the restricting HLA alleles and the presence of wild type epitopes, T-cell responses were only mounted against a few of the epitopes. Therefore, failure to detect Gag-specific T-cell responses is not primarily due to sequence variation within cognate epitopes. Furthermore, CD8+ T-cell-driven viral escape in this cohort was rare, although we cannot rule out escape in non-Gag regions where such mutations may be more common [3, 5]. Interestingly, participants with persisting detectable T-cell responses against invariant epitopes, had significantly lower viral load set point values when compared to participants with responses to epitopes containing variant sequences but subsequently lost these responses. These data suggest that persisting Gag-specific T-cell responses, which may be the result of continual invariant viral epitope presentation or the generation of *de novo* responses to arising variant epitopes, bear the greatest burden on control of early HIV-1 replication and disease progression [19, 24, 49].

Conclusions

The data indicate that broad and persistent Gag-specific effector and central memory type CD8+ T-cells are required for the maintenance of low viral load levels in primary infection. Acute and primary infection phase CD8+ T-cells in this study mostly failed to induce immune escape over the first year of infection, highlighting the need to better understand the characteristics of effective HIV-specific CD8+ T-cells. Our study suggests that an early and broadly directed Gag-specific CD8+ T-cell response in acute infection may augment early control, and may be informative for a rational approach for prophylactic and therapeutic vaccination.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We are grateful to the staff and management at St Mary's Hospital, Prince Mshiyeni Memorial Hospital, and all participating clinics in Durban, South Africa. We acknowledge Dr Johannes Viljoen and the Africa Center laboratory, Durban South Africa, for providing access to the sequencing facility.

B.D.W. and T.N. designed the study; they, M.R., K.G. and Z.M.N. designed the experiments; M.R., K.G., Z.M.N., L.M. and M.v.S. performed the experiments; M.J. and M.M. recruited participants and coordinated the HPP Acute HIV Infection cohort; M.R. and K.G. analyzed the data; M.R., B.D.W., and T.N. wrote the paper.

This research was funded by NIH (R37AI067073), the Bill and Melinda Gates Foundation, the South African Department of Science and Technology through the National Research Foundation and the International AIDS Vaccine Initiative. Partial funding was also received from the Victor Daitz Chair in HIV/TB Research and an International Early Career Scientist Award from the Howard Hughes Medical Institute to TN. MR work was funded by a UNESCO/L'Oréal Corporate Foundation fellowship and the Technology Innovation Agency of the Department of Science and Technology of South Africa. KG was the recipient of a Fogarty International Clinical Research Fellows award. Additional support was provided by the Mark and Lisa Schwartz Foundation.

References

1. Borrow P, Lewicki H, Hahn BH, Shaw GM, Oldstone MB. Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J Virol*. 1994; 68:6103–6110. [PubMed: 8057491]
2. 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. [PubMed: 8207839]
3. Goonetilleke N, Liu MKP, Salazar-Gonzalez JF, Ferrari G, Giorgi E, Ghanusov VV, et al. The first T cell response to transmitted/founder virus contributes to the control of acute viremia in HIV-1 infection. *J Exp Med*. 2009; 206:1253–1272. [PubMed: 19487423]
4. Pantaleo G, Demarest JF, Soudeyns H, Graziosi C, Denis F, Adelsberger JW, et al. Major expansion of CD8+ T cells with a predominant V beta usage during the primary immune response to HIV. *Nature*. 1994; 370:463–467. [PubMed: 8047166]
5. Liu MK, Hawkins N, Ritchie AJ, Ghanusov VV, Whale V, Brackenridge S, et al. Vertical T cell immunodominance and epitope entropy determine HIV-1 escape. *J Clin Invest*. 2013; 123:380–393. [PubMed: 23221345]
6. Huber M, Trkola A. Humoral immunity to HIV-1: neutralization and beyond. *J Intern Med*. 2007; 262:5–25. [PubMed: 17598812]
7. Radebe M, Nair K, Chonco F, Bishop K, Wright JK, van der Stok M, et al. Limited Immunogenicity of HIV CD8+ T-Cell Epitopes in Acute Clade C Virus Infection. *J Infect Dis*. 2011; 204:768–776. [PubMed: 21844303]
8. Fischer W, Ghanusov VV, Giorgi EE, Hraber PT, Keele BF, Leitner T, et al. Transmission of single HIV-1 genomes and dynamics of early immune escape revealed by ultra-deep sequencing. *PLoS One*. 2010; 5:e12303. [PubMed: 20808830]
9. Betts MR, Ambrozak DR, Douek DC, Bonhoeffer S, Brenchley JM, Casazza JP, et al. Analysis of total human immunodeficiency virus (HIV)-specific CD4(+) and CD8(+) T-cell responses: relationship to viral load in untreated HIV infection. *J Virol*. 2001; 75:11983–11991. [PubMed: 11711588]
10. Addo MM, Yu XG, Rathod A, Cohen D, Eldridge RL, Strick D, et al. Comprehensive epitope analysis of human immunodeficiency virus type 1 (HIV-1)-specific T-cell responses directed against the entire expressed HIV-1 genome demonstrate broadly directed responses, but no correlation to viral load. *J Virol*. 2003; 77:2081–2092. [PubMed: 12525643]
11. Gray CM, Mlotshwa M, Riou C, Mathebula T, de Assis Rosa D, Mashishi T, et al. Human Immunodeficiency Virus-Specific Gamma Interferon Enzyme-Linked Immunospot Assay Responses Targeting Specific Regions of the Proteome during Primary Subtype C Infection Are Poor Predictors of the Course of Viremia and Set Point. *J Virol*. 2009; 83:470–478. [PubMed: 18945774]
12. Chen H, Piechocka-Trocha A, Miura T, Brockman MA, Julg BD, Baker BM, et al. Differential neutralization of human immunodeficiency virus (HIV) replication in autologous CD4 T cells by HIV-specific cytotoxic T lymphocytes. *J Virol*. 2009; 83:3138–3149. [PubMed: 19158248]
13. Ferrari G, Korber B, Goonetilleke N, Liu MK, Turnbull EL, Salazar-Gonzalez JF, et al. Relationship between functional profile of HIV-1 specific CD8 T cells and epitope variability with

the selection of escape mutants in acute HIV-1 infection. *PLoS Pathog.* 2011; 7:e1001273. [PubMed: 21347345]

14. Perreau M, Levy Y, Pantaleo G. Immune response to HIV. *Curr Opin HIV AIDS.* 2013; 8:333–340. [PubMed: 23743723]
15. Draenert R, Verrill CL, Tang Y, Allen TM, Wurcel AG, Boczanowski M, et al. Persistent recognition of autologous virus by high-avidity CD8 T cells in chronic, progressive human immunodeficiency virus type 1 infection. *J Virol.* 2004; 78:630–641. [PubMed: 14694094]
16. Koibuchi T, Allen TM, Lichterfeld M, Mui SK, O'Sullivan KM, Trocha A, et al. Limited sequence evolution within persistently targeted CD8 epitopes in chronic human immunodeficiency virus type 1 infection. *J Virol.* 2005; 79:8171–8181. [PubMed: 15956562]
17. Saez-Cirion A, Sinet M, Shin SY, Urrutia A, Versmisse P, Lacabartz C, et al. Heterogeneity in HIV suppression by CD8 T cells from HIV controllers: association with Gag-specific CD8 T cell responses. *J Immunol.* 2009; 182:7828–7837. [PubMed: 19494307]
18. Julg B, Williams KL, Reddy S, Bishop K, Qi Y, Carrington M, et al. Enhanced anti-HIV functional activity associated with Gag-specific CD8 T-cell responses. *J Virol.* 2010
19. Perez CL, Milush JM, Buggert M, Eriksson EM, Larsen MV, Liegler T, et al. Targeting of conserved gag-epitopes in early HIV infection is associated with lower plasma viral load and slower CD4(+) T cell depletion. *AIDS Res Hum Retroviruses.* 2013; 29:602–612. [PubMed: 23140171]
20. 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. [PubMed: 17173051]
21. Turk G, Ghiglione Y, Falivene J, Socias ME, Laufer N, Coloccini RS, et al. Early Gag Immunodominance of the HIV-Specific T-Cell Response during Acute/Early Infection Is Associated with Higher CD8+ T-Cell Antiviral Activity and Correlates with Preservation of the CD4+ T-Cell Compartment. *J Virol.* 2013; 87:7445–7462. [PubMed: 23616666]
22. Brumme ZL, Brumme CJ, Carlson J, Streeck H, John M, Eichbaum Q, et al. Marked epitope- and allele-specific differences in rates of mutation in human immunodeficiency type 1 (HIV-1) Gag, Pol, and Nef cytotoxic T-lymphocyte epitopes in acute/early HIV-1 infection. *J Virol.* 2008; 82:9216–9227. [PubMed: 18614631]
23. Streeck H, Jolin JS, Qi Y, Yassine-Diab B, Johnson RC, Kwon DS, et al. Human immunodeficiency virus type 1-specific CD8+ T-cell responses during primary infection are major determinants of the viral set point and loss of CD4+ T cells. *J Virol.* 2009; 83:7641–7648. [PubMed: 19458000]
24. Mlotshwa M, Riou C, Chopera D, de Assis Rosa D, Ntale R, Treunicht F, et al. Fluidity of HIV-1-specific T-cell responses during acute and early subtype C HIV-1 infection and associations with early disease progression. *J Virol.* 2010; 84:12018–12029. [PubMed: 20826686]
25. Henn MR, Boutwell CL, Charlebois P, Lennon NJ, Power KA, Macalalad AR, et al. Whole genome deep sequencing of HIV-1 reveals the impact of early minor variants upon immune recognition during acute infection. *PLoS Pathog.* 2012; 8:e1002529. [PubMed: 22412369]
26. Freel SA, Saunders KO, Tomaras GD. CD8(+)/T-cell-mediated control of HIV-1 and SIV infection. *Immunol Res.* 2011; 49:135–146. [PubMed: 21170741]
27. McMichael AJ, Borrow P, Tomaras GD, Goonetilleke N, Haynes BF. The immune response during acute HIV-1 infection: clues for vaccine development. *Nat Rev Immunol.* 2010; 10:11–23. [PubMed: 20010788]
28. Lichterfeld M, Yu XG, Cohen D, Addo MM, Malenfant J, Perkins B, et al. HIV-1 Nef is preferentially recognized by CD8 T cells in primary HIV-1 infection despite a relatively high degree of genetic diversity. *AIDS.* 2004; 18:1383–1392. [PubMed: 15199314]
29. Jones NA, Wei X, Flower DR, Wong M, Michor F, Saag MS, et al. Determinants of human immunodeficiency virus type 1 escape from the primary CD8+ cytotoxic T lymphocyte response. *J Exp Med.* 2004; 200:1243–1256. [PubMed: 15545352]
30. Allen TM, Altfeld M, Geer SC, Kalife ET, Moore C, O'Sullivan KM, et al. Selective escape from CD8+ T-cell responses represents a major driving force of human immunodeficiency virus type 1

- (HIV-1) sequence diversity and reveals constraints on HIV-1 evolution. *J Virol.* 2005; 79:13239–13249. [PubMed: 16227247]
31. Kawashima Y, Pfafferoth K, Frater J, Matthews P, Payne R, Addo M, et al. Adaptation of HIV-1 to human leukocyte antigen class I. *Nature.* 2009; 458:641–645. [PubMed: 19242411]
 32. Schellens IM, Navis M, van Deutekom HW, Boeser-Nunnink B, Berkhout B, Kootstra N, et al. Loss of HIV-1-derived cytotoxic T lymphocyte epitopes restricted by protective HLA-B alleles during the HIV-1 epidemic. *AIDS.* 2011; 25:1691–1700. [PubMed: 21681058]
 33. Goepfert PA, Lumm W, Farmer P, Matthews P, Prendergast A, Carlson JM, et al. Transmission of HIV-1 Gag immune escape mutations is associated with reduced viral load in linked recipients. *J Exp Med.* 2008; 205:1009–1017. [PubMed: 18426987]
 34. Wright JK, Novitsky V, Brockman MA, Brumme ZL, Brumme CJ, Carlson JM, et al. Influence of Gag-protease-mediated replication capacity on disease progression in individuals recently infected with HIV-1 subtype C. *J Virol.* 2011; 85:3996–4006. [PubMed: 21289112]
 35. Thobakgale CF, Ramduth D, Reddy S, Mkhwanazi N, de Pierres C, Moodley E, et al. Human immunodeficiency virus-specific CD8+ T-cell activity is detectable from birth in the majority of in utero-infected infants. *J Virol.* 2007; 81:12775–12784. [PubMed: 17881456]
 36. Streeck H, Frahm N, Walker BD. The role of IFN-gamma Elispot assay in HIV vaccine research. *Nat Protoc.* 2009; 4:461–469. [PubMed: 19282851]
 37. Novitsky V, Rybak N, McLane MF, Gilbert P, Chigwedere P, Klein I, et al. Identification of human immunodeficiency virus type 1 subtype C Gag-, Tat-, Rev-, and Nef-specific elispot-based cytotoxic T-lymphocyte responses for AIDS vaccine design. *J Virol.* 2001; 75:9210–9228. [PubMed: 11533184]
 38. Ngumbela KC, Day CL, Mncube Z, Nair K, Ramduth D, Thobakgale C, et al. Targeting of a CD8 T cell env epitope presented by HLA-B*5802 is associated with markers of HIV disease progression and lack of selection pressure. *AIDS Res Hum Retroviruses.* 2008; 24:72–82. [PubMed: 18275350]
 39. Llano, A.; Williams, A.; Overa, A.; Silva-Arrieta, S.; Brander, C. HIV Molecular Immunology. Los Alamos, New Mexico: Theoretical Biology and Biophysics; 2013. Best-Characterized HIV-1 CTL Epitopes: The 2013 Update; p. 3
 40. Ndhlovu ZM, Proudfoot J, Cesa K, Alvino DM, McMullen A, Vine S, et al. Elite controllers with low to absent effector CD8+ T cell responses maintain highly functional, broadly directed central memory responses. *J Virol.* 2012; 86:6959–6969. [PubMed: 22514340]
 41. Calarota SA, Foli A, Maserati R, Baldanti F, Paolucci S, Young MA, et al. HIV-1-specific T cell precursors with high proliferative capacity correlate with low viremia and high CD4 counts in untreated individuals. *J Immunol.* 2008; 180:5907–5915. [PubMed: 18424710]
 42. Chopera DR, Woodman Z, Mlisana K, Mlotshwa M, Martin DP, Seoighe C, et al. Transmission of HIV-1 CTL escape variants provides HLA-mismatched recipients with a survival advantage. *PLoS Pathog.* 2008; 4:e1000033. [PubMed: 18369479]
 43. Turnbull EL, Wong M, Wang S, Wei X, Jones NA, Conrod KE, et al. Kinetics of expansion of epitope-specific T cell responses during primary HIV-1 infection. *J Immunol.* 2009; 182:7131–7145. [PubMed: 19454710]
 44. Pala P, Serwanga J, Watera C, Ritchie AJ, Moodie Z, Wang M, et al. Quantitative and qualitative differences in the T cell response to HIV in uninfected Ugandans exposed or unexposed to HIV infected partners. *J Virol.* 2013
 45. McDermott AB, Koup RA. CD8(+) T cells in preventing HIV infection and disease. *AIDS.* 2012; 26:1281–1292. [PubMed: 22441256]
 46. 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. [PubMed: 16501126]
 47. Geldmacher C, Currier JR, Herrmann E, Haule A, Kuta E, McCutchan F, et al. CD8 T-cell recognition of multiple epitopes within specific Gag regions is associated with maintenance of a low steady-state viremia in human immunodeficiency virus type 1-seropositive patients. *J Virol.* 2007; 81:2440–2448. [PubMed: 17182686]

48. 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. [PubMed: 15016844]
49. Wang YE, Li B, Carlson JM, Streeck H, Gladden AD, Goodman R, et al. Protective HLA class I alleles that restrict acute-phase CD8+ T-cell responses are associated with viral escape mutations located in highly conserved regions of human immunodeficiency virus type 1. *J Virol.* 2009; 83:1845–1855. [PubMed: 19036810]
50. Lichterfeld M, Kaufmann DE, Yu XG, Mui SK, Addo MM, Johnston MN, et al. Loss of HIV-1-specific CD8+ T cell proliferation after acute HIV-1 infection and restoration by vaccine-induced HIV-1-specific CD4+ T cells. *J Exp Med.* 2004; 200:701–712. [PubMed: 15381726]
51. Burgers WA, Riou C, Mlotshwa M, Maenetje P, de Assis Rosa D, Brenchley J, et al. Association of HIV-specific and total CD8+ T memory phenotypes in subtype C HIV-1 infection with viral set point. *J Immunol.* 2009; 182:4751–4761. [PubMed: 19342652]
52. Champagne P, Ogg GS, King AS, Knabenhans C, Ellefsen K, Nobile M, et al. Skewed maturation of memory HIV-specific CD8 T lymphocytes. *Nature.* 2001; 410:106–111. [PubMed: 11242051]
53. Northfield JW, Loo CP, Barbour JD, Spotts G, Hecht FM, Klenerman P, et al. Human immunodeficiency virus type 1 (HIV-1)-specific CD8+ T(EMRA) cells in early infection are linked to control of HIV-1 viremia and predict the subsequent viral load set point. *J Virol.* 2007; 81:5759–5765. [PubMed: 17376902]
54. Addo MM, Draenert R, Rathod A, Verrill CL, Davis BT, Gandhi RT, et al. Fully differentiated HIV-1 specific CD8+ T effector cells are more frequently detectable in controlled than in progressive HIV-1 infection. *PLoS One.* 2007; 2:e321. [PubMed: 17389912]

Figure 1.

Kinetics of *ex vivo* IFN- γ ELISPOT HIV-1-specific CD8+ T-cell responses during the 1 year follow-up period. (A) Frequency of T-cell recognition across the entire HIV-1 subtype C proteome. The bars represent response frequencies to Nef, Pol, Gag, Env, VVTRV (Vpr, Vpu, Rev, Tat and Vif) proteins from 4 to 52 weeks post infection; (B) Bar graphs depicting the mean magnitudes and relative contributions of Nef, Gag, Pol, Env, and VVTRV proteins to the total magnitude and (C) total number of epitopes breadth of HIV-1-specific T-cell responses at the specified time points. (D–H) Longitudinal characterization of HIV-1-specific CD8+ T-cell responses; (D) breadth of Nef, Pol (E), Gag (F), Env (G) and VVTRV (H) specific IFN- γ ELISPOT CD8+ T-cell responses measured from 4 to 52 weeks post infection in a cohort of 18 subjects. Each symbol represents the total number of epitopes targeted per participant.

Figure 2.

Association between HIV-1-specific CD8⁺ T-cell responses and viral load. (A) Spearman correlation between the breadth of Nef-specific responses and viral load set point at 8 weeks; (B–D) correlations between the breadth of Gag-specific responses and viral load set point at 8, 26, and 52 weeks post infection.

Figure 3.

The impact of persistent T-cell responses measured between 4 weeks and 52 weeks post infection on viral load set point. (A) A lack of association between persistent responses targeting epitopes within all proteins and viral load set point; (B) A lack of association between persistent responses targeting epitopes within Pol and viral load set point; (C) A significant negative association between the breadth of Gag-specific persistent T-cell responses and viral load set point; (D) A significant negative association between the breadth of Gag-specific cultured ELISPOT T-cell responses and viral load set point.

Figure 4. Recognition patterns associated with wild type and variant Gag sequences at 2–4 weeks and 52 weeks post infection. (A) The actual total number of wild type/variant sequences and the actual total (cumulative) number of T-cell responses measured (B) T-cell recognition in relation to wild type Gag sequences shown as the magnitudes of IFN- γ ELISPOT responses at 2–4 weeks and 52 weeks post infection. (C) T-cell recognition in relation to sequence variations in Gag at 2–4 weeks and 52 weeks post infection. Each line represents the specific epitope targeted. The sequences of the targeted epitopes at 2–4 weeks and 52 weeks post infection are shown underneath the graphs. The underlined areas indicate the putative targeted epitope.

Confirmed persistent HLA class I-restricted HIV-specific CD8+ T-cell epitopes and overlapping peptides targeted between 6–52 weeks post infection

Table 1

Participant ID	Epitopes and OLPs targeted: between 6–52 weeks post infection		Locations of the targeted epitopes:								Viral load set point (Log ₁₀ copies/ml)
	Defined HLA class I restricted epitopes	OLPs	Gag	Pol	Nef	Vif	Vpr	Env	Rev	Tat	
AS1-0703	none detected		1	1							4.89
AS1-0876	B1503-VF9(p24)		1								3.61
AS1-0919	A29-YY8(Nef)	#202(RT)		1	1						4.44
AS2-0016	A3002-KIY9(Int), B42-FL9(Vpr), B42-YL9(RT), B42-RM9(Nef)	#275(Int)		3	1		1				4.70
AS2-1037	B1510-IL9(Rev)								1		5.54
AS2-0174	A3002-AY11(RT), A3002-KIY9(Int), Cw3-YL9(p24)		1	2							3.20
AS2-0184	A23-RW8(Nef)				1						5.16
AS2-0341	None detected	#81(Nef), #408(Vif)			1	1					5.18
AS2-0358	A23-HW9(p17), A23-RW8(Nef), B42-RL10(Rev), B53-EW10(RT)		1	1	1				1		4.79
AS2-0483	B42-TL9(p24)		1								5.20
AS2-0802	A3002-RY11(p17)		1								4.40
AS2-0945	B57-TW10(p24), B1510-IL9(Rev)		1						1		5.61
AS3-0268	A29-SY9(gp160)								1		5.57
AS3-0369	A66-ER11(RT), Cw3-YL9(p24)		1	1							3.78
AS3-0458	A29-SY9(gp160), B44-AW11(p24), B57-FF9(RT), B57-KI13(RT), Cw4-EW11(gp120)		1	2					2		3.97
AS3-0740	B44-AW11(p24), B44-IW11(RT)	#78(Nef)	1	1	1						3.38
AS5-0643	A2-SL9(p17), A2-AL9(Vpr), B42-YL9(RT), Cw0202-EY11(RT)		1	2			1				4.67
AS5-0953	A3-RLY10(p17)		1								3.62