Correlates of T-cell mediated viral control and phenotype of CD8+ T-cells in HIV-2, a
naturally contained human retroviral infection.

Thushan I. de Silva1,2,7, Yanchun Peng6, Aleksandra Leligdowicz1,2, Irfan Zaidi1, Lucy Li1, Harry
Griffin2, Marie-Eve Blais2, Tim Vincent1,3, Mavinga Saraiva1,3, Louis-Marie Yindom2, Carla van
Tienen1, Philippa Easterbrook5, Assan Jaye1, Hilton Whittle1,4, Tao Dong6 and Sarah L. Rowland-
Jones2.

1. Medical Research Council (UK) Laboratories the Gambia, Atlantic Road, PO Box 273,
   Fajara, The Gambia, West Africa.
2. Nuffield Department of Medicine, Weatherall Institute of Molecular Medicine, John
   Radcliffe Hospital, Headington, Oxford, UK.
3. Projecto de Saúde de Bandim/Indepth Network, Apartado 861, 1004 Bissau Codex, Guinea-
   Bissau.
4. Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical
   Medicine, London, UK.
5. Department of Infectious Diseases, Nelson Mandela School of Medicine, University of
   KwaZulu Natal, South Africa.
6. MRC Human Immunology Unit, Weatherall Institute of Molecular Medicine, John Radcliffe
   Hospital, Oxford, UK.
7. Academic Unit of Immunology and Infectious Diseases, Department of Infection and
   Immunity, The University of Sheffield Medical School, Sheffield, UK.
KEY POINTS

- HIV-2 viral control is associated with a polyfunctional Gag-specific CD8+ T-cell response, but not perforin upregulation.
- Our findings provide insight into cellular immune responses associated with a naturally contained human retroviral infection.

ABSTRACT

While a significant proportion of HIV-2-infected individuals are asymptomatic and maintain undetectable viral loads (controllers), 15-20% progress to AIDS and are predicted by detectable viraemia. Identifying immune correlates that distinguish these two groups should provide insights into how a potentially pathogenic retrovirus can be naturally controlled. We performed a detailed study of HIV-2-specific cellular responses in a unique community cohort in Guinea-Bissau followed for over two decades. T-cell responses were compared between controllers (n = 33) and viraemic subjects (n = 27) using overlapping peptides, MHC class I tetramers and multi-parameter flow cytometry. HIV-2 viral control was significantly associated with a high magnitude, polyfunctional Gag-specific CD8+ T-cell response, but not with greater perforin upregulation. This potentially protective HIV-2-specific response is surprisingly narrow. HIV-2 Gag-specific CD8+ T-cells are at an earlier stage of differentiation than CMV-specific CD8+ T-cells, do not contain high levels of cytolytic markers and exhibit low levels of activation and proliferation, representing distinct properties from CD8+ T-cells associated with HIV-1 control. These data reveal the potential T-cell correlates of HIV-2 control and the detailed phenotype of virus-specific CD8+ T-cells in a naturally contained retroviral infection.
INTRODUCTION

Nearly three decades after the identification of HIV-1, considerable progress has been made in understanding the mechanisms by which HIV evades and profoundly damages the immune system, but a vaccine that reliably prevents or controls HIV remains elusive. Important insights into correlates of protective immunity can be gained from studying SIV in animal models and human subjects infected with HIV-1 who do not develop immunodeficiency. One such model that has not been adequately investigated is infection with HIV-2. HIV-1 and HIV-2 share 30-60% sequence identity and show many similarities in their cytopathic potential and replication kinetics in vitro\(^1\). A notable difference is the presence of Vpx in HIV-2, with possible implications for enhanced infection of dendritic cells (and therefore greater antigen presentation) in HIV-2 due to Vpx-mediated antagonism of the host restriction factor SAMHD1\(^2\). Clinically, HIV-2 differs from HIV-1 in that a substantial proportion of infected people (approx. 37% in our cohort) maintain an undetectable plasma viral load (VL) for over a decade with no signs of immunodeficiency\(^3\). Unlike the rare phenomenon of “elite control” in HIV-1 infection, this phenotype is both clinically stable and occurs more commonly than disease progression. Nevertheless, those with HIV-2 who develop disease (15-20%) do so in a manner\(^4\) and time-frame\(^5\) that cannot be distinguished clinically from HIV-1 infection. Identifying the key differences between these two groups at either end of the HIV-2 clinical spectrum should provide insights into the mechanisms by which humans can control infection with a potentially pathogenic retrovirus. Furthermore, recent data has suggested that preceding HIV-2 infection can limit the rate of disease progression and HIV-1 viral evolution following HIV-1 super-infection\(^6\). As cross-reactive HIV-2 Gag-specific cytotoxic T-lymphocyte (CTL) responses\(^7\) are a possible explanation for these observations, characterization of CD8+ T-cells in HIV-2-infected subjects could aid our understanding of what would be required of a vaccine that could successfully contain HIV-1 via induction of T-cell responses.
We have previously shown that HIV-2-infected subjects with high CD4+ counts are more likely to preserve polyfunctional virus-specific T-cell responses than their HIV-1-infected counterparts, but whether these responses distinguish HIV-2 controllers from viraemic progressors is not known. When the T-cell response towards the HIV-2 proteome was compared between these two groups, viral control was strongly associated with high magnitude HIV-2 Gag-specific responses. However, this study was performed using ELISpot assays that did not distinguish between CD4+ and CD8+ responses and further detailed phenotypic and functional characterization of this potentially protective response is lacking. Here we present data from a study of HIV-2 Gag-specific T-cell responses from subjects selected to represent both controllers and viraemic subjects, in order to identify the cellular immune correlates of HIV-2 viral control. Our study arguably represents the most comprehensive analysis of HIV-2-specific T-cells to date, including detailed ex vivo phenotypic analysis of CD8+ T-cells targeting several immunodominant epitopes and direct comparison of both cytokine-mediated and cytotoxic functional capacity in HIV-2 controllers and viraemic subjects.

**MATERIALS AND METHODS**

**Study subjects**

Antiretroviral therapy (ART) naïve HIV-2 mono-infected subjects from the Caió community cohort in rural Guinea-Bissau were recruited following informed consent. This cohort was established in 1989 and has followed HIV-2 infected individuals identified via community serosurveys for over two decades. CD4+ T-cell subset analysis was performed using fresh whole blood staining with two-colour immunofluorescence reagents (anti-CD45/anti-CD4) and a CyFlow-SL flow cytometer (Partec). Peripheral blood mononuclear cells (PBMCs) were cryopreserved. Confirmation of HIV-2 status was undertaken using a diagnostic algorithm involving both serological and molecular
methods. HIV-2 plasma VLs were quantified using an in-house RT-PCR assay as previously described (lower limit of detection 100 copies/ml). Ethical approval was provided by the joint MRC/Gambia Government Ethics Committee and the Guinea-Bissau Ministry of Health. HLA class I genotyping on subjects in this cohort was performed during previous studies using sequencing. This study was conducted in accordance with the Declaration of Helsinki.

**Peptide-MHC class I tetramers**

Generation of peptide/HLA class I tetramers conjugated to streptavidin-PE (Sigma-Aldrich) was performed according to previously described protocols. The tetramers used were: HLA-B*3501-NPVPVGNIY, B*5801-TSTVDEQIQW, B*5301-TPYDINQML, B14-DRFYKSLRA (HIV-2 Gag p26); A*0201-NLVPMVATV (CMV pp65), B35-EPLPQGQLTAY and B8-RAKFKQLL (EBV BZLF1).

**Antibodies**

The following directly conjugated monoclonal antibodies were used: CD107a-PECy5, CD4-APCefluor780, IFN-γ-PECy7, TNF-α-efluor450, IL-2-PE, CD27-APCe780, CD160-efluor647 (Ebiscience); CD8-ECD (Beckman Coulter); Perforin-FITC (clone D48, Gen-Probe); CD3-Qdot655, CD4-Qdot605, CD8-Qdot705, CD45RO-TexasRedPE (Invitrogen); CD14-V500, CD19-V500, Granzyme B-Alexafluor700, Ki67-Alexafluor488, Bcl-2-V450 (BD Biosciences); CD38-APC, 2B4(CD244)-PECy5.5 (clone C1.7), PD-1-PECy7 (clone EH12.2H7), T-bet-Brilliant Violet (clone 4B10), HLA-DR-Alexafluor700 (Biolegend). LIVE/DEAD® fixable Aqua Dead Cell Stain (for 405nm excitation) was obtained from Invitrogen. Unconjugated CD57 MAb (clone NK-1, BD Biosciences) was conjugated in our laboratory to Quantum dot 565 obtained from Invitrogen.
Intracellular cytokine stimulation and polychromatic flow cytometry

Assays were performed at the MRC Laboratories in the Gambia and cells analyzed using a CyAn flow cytometer (Dako, Beckman Coulter). Overlapping peptide sets representing entire HIV-2 Gag (n = 70) and Env (n = 107) proteins were used for stimulations. Freshly thawed, cryopreserved PBMCs were resuspended in RPMI supplemented with 10% heat-inactivated fetal calf serum (FCS), 2mM L-glutamine (Sigma-Aldrich), 1% PenStrep (Sigma-Aldrich) (R10) and 60µg/ml DNase solution (Type IV, Sigma-Aldrich) for 15 minutes at 37°C. Cells were washed and resuspended in R10 and rested overnight at a concentration of 10⁶/ml, after which 5 x 10⁵ live PBMCs (determined by trypan blue exclusion) were stimulated with overlapping Gag (and where adequate cells were available, Env) peptide pools at 2µg/ml (each peptide) and co-stimulatory antibodies anti-CD28 and anti-CD49 (BD Biosciences) at 1µg/ml. Cells stimulated with Staphylococcus Enterotoxin B (SEB, Sigma) at 2µg/ml and co-stimulatory antibodies alone were also included. Following incubation for 1 hour at 37°C, Brefeldin A (3µg/ml) and Monensin (2µM) (Ebioscience) and surface stain for CD107a were added. Following a further 5-hour incubation, cells were stained with Aqua, CD4 and CD8 antibodies. Cells were then fixed and permeabilized (Cytofix/Cytoperm™, BD Biosciences) and stained intracellularly for IFN-γ, TNF-α, IL-2 and perforin. Perforin staining was excluded in an additional SEB stimulated condition utilized for flow-minus-one (FMO) staining to aid with subsequent gating.

Electronic compensation was performed using the BD CompBead Anti-Mouse Ig set (BD Biosciences) and antibodies described above. Between 300,000 and 400,000 events were acquired for each sample. Data analysis was performed using FlowJo version 9.1 (Treestar). Forward scatter (FSC)-area versus FSC-height parameters were used to exclude cell aggregates, followed by exclusion of dead cells and gating on lymphocytes using FSC and side scatter (SSC) properties (Fig S1). CD4+ and CD8+ T-cells were identified. Boolean/combinatorial gate arrays were created using
each measured marker (CD107a, IFN-γ, TNF-α, IL-2 and perforin), which determined the frequency of each response pattern based on all possible combinations. Responses detected in the negative (anti-CD28/anti-CD49d) control were subtracted from responses observed in stimulated samples for each response individually prior to further analysis. In cases where the stimulated sample value is smaller than that of the anti-CD28/anti-CD49d control, negative values arise. These were set to zero. To avoid systematic bias with this approach, a threshold was set below which all positive values were also zeroed on the assumption that the distribution of background values is symmetric around zero\textsuperscript{16}. This threshold (0.009 for the CD8+ and 0.01 for the CD4+ dataset) was chosen based on the distribution of negative values for each dataset of twice the 90th centile.

**Tetramer staining and polychromatic flow cytometry**

Assays were performed at the Weatherall Institute of Molecular Medicine (Oxford) and cells analyzed with an LSRII flow cytometer (BD). Cryopreserved PBMCs were thawed as described above. 1 x 10\textsuperscript{6} live PBMCs were stained with Aqua for 30min at 4°C followed by 1µg of tetramer-PE for 15min at 37°C. Surface staining with CD3-Qdot655, CD4-Qdot605, CD8-Qdot705, CD14-V500, CD19-V500, CD27-efluor780, CD45RO-TexasRedPE, CD57-Qdot565 and PD-1-PECy7, as well as 2B4-PECy5.5 and CD160-efluor647 in panel-1, and CD38-APC and HLA-DR-Alexafluor700 in panel-2 were performed. Following fixing and permeabilization, intracellular staining with Perforin-FITC, Granzyme B-Alexafluor700 and T-bet-Brilliant Violet in panel-1 and Ki67-Alexafluor488 and Bcl-2-V450 in panel-2 were done. FMO samples were included to aid gating during subsequent flow cytometric analysis.

Electronic compensation was performed as described above. Between 500,000 and 1,000,000 events were acquired for each sample. FSC area and FSC height were used to exclude cell aggregates, followed by removal of CD14+, CD19+, dead cells and gating on CD3+ cells. FSC and
SSC were then used to gate on small lymphocytes, followed by isolation of CD8+ cells and tetramer-staining cells (Fig S2). Boolean/combinatorial gate arrays were created to measure the co-expression of PD-1, 2B4 and CD160.

**Statistical analysis**

Statistical analyses were performed using Graphpad Prism version 5.0 for Macintosh (GraphPad Software). Comparisons between variables were performed using the Mann-Whitney U test, Kruskal-Wallis test with Dunn’s post test for multiple comparisons or the Wilcoxon matched pairs test. Discrete data were compared by the chi-square test or Fisher’s exact test. Correlations between non-normally distributed data were made using the Spearman’s rank correlation coefficient.

**RESULTS**

**Study subjects**

Sixty HIV-2 mono-infected individuals were recruited. Clinical and demographic parameters are described in Table 1. HIV-2 controllers were defined as asymptomatic individuals with a current undetectable VL (<100 copies/ml). The predominantly female and older age of the subjects may be due to an increased risk of HIV-2 acquisition in older women\(^\text{17}\) and higher non-HIV-related mortality in men in sub-Saharan Africa\(^\text{18}\).

**HIV-2 control is significantly associated with greater magnitude and polyfunctionality of the Gag-specific CD8+ T-cell response**

The magnitude of the CD8+ (but not CD4+) Gag-specific IFN-\(\gamma\) response differentiated HIV-2 controllers from viraemic individuals (\(p = 0.008\), Fig 1a and b). Although the magnitude of
polyfunctional CD4+ T-cells was similar between the two groups, the proportion of the total CD4+ response expressing \( \geq 3 \) functions was significantly higher in HIV-2 controllers (Fig 1c and d). A greater proportion of the Gag-specific CD8+ response consisted of IFN-\( \gamma \) and TNF-\( \alpha \) producing cells in controllers (Fig 2a). The frequency of polyfunctional CD8+ T cells was significantly higher in controllers (Fig 2b) and polyfunctionality correlated inversely with plasma VL (Fig 2c). In contrast, the proportion of the CD8+ T-cell response made up of mono-functional cells increased with higher VL (Fig 2d). No significant association was observed between CD4+ count and the CD8+ IFN-\( \gamma \) response or polyfunctionality (Fig S3). Using median fluorescence intensity (MFI) of each parameter as a proxy for the amount of cytokine produced per cell, we also observed, as previously described\(^{10} \), that polyfunctional Gag-specific CD8+ T-cells secreted more IFN-\( \gamma \) (Fig S4) and TNF-\( \alpha \), but not CD107a or IL-2 (data not shown) than their ‘less functional’ counterparts.

HIV-2 Env-specific responses (measured in 25 HIV-2 controllers and 14 viraemic subjects) were almost exclusively from CD8+ T-cells (data not shown). No significant difference in the magnitude of the Env-specific CD8+ IFN-\( \gamma \) response was observed between the two groups (Fig S5). Gag-specific CD8+ responses were more polyfunctional than Env-specific responses, although this difference was primarily found in HIV-2 controllers (Fig S6).

**The HIV-2 Gag-specific CD8+ T-cell response is highly focused and often absent in viraemic individuals**

Peptide-MHC class I tetramers specific for four immunodominant HIV-2 Gag epitopes in this cohort\(^ {11} \) were used to determine the phenotype of HIV-2-specific CD8+ T-cells (HLA B53, B58, B35 and B14-restricted). CTLs targeting two of these epitopes (B35 and B58-restricted) are also known to recognise the equivalent regions in HIV-1\(^ {17} \). Thirty-one individuals with B53, B58, B35 or B14 alleles were included (Table S1, a total of 35 tetramer-staining experiments). Also included
were five CMV-specific and eight EBV-specific tetramer-staining populations. Frequencies of all tetramer-staining populations are detailed in Table S2.

A greater proportion of HIV-2 controllers compared to viraemic subjects had tetramer-staining populations (84% vs. 42%, \( p = 0.02 \), Fig 3b). This is an important observation but prevented a meaningful comparison of HIV-2 specific CD8+ T-cell phenotype between the two groups and therefore all HIV-2 populations were analysed together (25 tetramer-staining populations from 21 HIV-2-infected individuals: 16 controllers, five viraemic). We observed a strong correlation \( (r = 0.71) \) between the magnitude of the CD8+ IFN-\( \gamma \) response to the total Gag pool (by ICS) and the % of tetramer-staining T-cells in each subject (Fig 3c), suggesting that the bulk of the Gag-specific response in these subjects is accounted for by CD8+ T-cells targeting one or more of these epitopes. We then reanalyzed previously reported IFN-\( \gamma \) ELISpot data from the same cohort, generated using a 3D-peptide matrix. The total Gag pool-specific magnitude was significantly greater in individuals who responded to \( \geq 1 \) of these four HIV-2 Gag epitopes (40/64, 62.5%) than to those who did not (Fig 3d): median (IQR) 1103 (524 – 2081) SFU/ml vs. 195 (0 – 481) SFU/ml, thus confirming the remarkable immunodominance of these epitopes. Furthermore, of the individuals who displayed B53, B58, B35 or B14-restricted responses, 32/40 (80%) responded to only one of these peptides. This represents an incredibly restricted breadth in the HIV-2-specific Gag response shown to be associated with viraemic control.

**Control of HIV-2 viraemia is not associated with CD8+ T-cells with high cytotoxic potential**

HIV-1 control is associated with the capacity of HIV-1-specific CD8+ T-cells to express perforin on antigen stimulation, as well as enrichment of other cytolytic markers. We explored whether similar qualities are required for HIV-2 control. Although perforin co-expression (with one or more of IFN-\( \gamma \), TNF-\( \alpha \), IL-2 and CD107a) was readily identifiable following both SEB and Gag
stimulation, this did not differentiate HIV-2 controllers from viraemic subjects (Fig 4a and 4b). We also assessed the degree of *ex vivo* perforin, granzyme B and T-bet expression in virus-specific CD8+ T-cells. When compared to bulk CD8+ T-cells, HIV-2-specific T-cells showed modest increases in granzyme B, similar expression of T-bet and lower levels of perforin (Fig 4c). Expression levels on viraemic donor cells did not cluster together. CMV-specific CD8+ T-cells displayed significantly higher granzyme B and T-bet expression than HIV-2 and EBV-specific T-cells (Fig 4d). Analysis of T-bet MFI revealed a similar picture (data not shown).

**HIV-2-specific CD8+ T-cells display an earlier differentiated phenotype**

Previous studies have shown significant heterogeneity in the phenotype of CD8+ T-cells specific for different chronic viral infections. HIV-2-specific CD8+ T-cells displayed greater CD27 expression than CMV-specific CD8+ T-cells (Fig 5a and 5c), representing T-cells at an earlier stage of differentiation. No clear pattern was observed in controllers vs. viraemic donor cells (Fig 5a). While CMV-specific T-cells tended to display a ‘terminal effector’ phenotype of CD27-/CD45RO-, HIV-2-specific and EBV-specific T-cells were more frequently CD27+/CD45RO+ (Fig 4b and 4c). No significant difference was seen in antigen-specific CD8+ T-cell senescence based on expression of CD57 (data not shown).

**HIV-2-specific CD8+ T-cells display high levels of PD-1, 2B4 and CD160 expression**

Recent data have defined a relationship between co-expression of multiple inhibitory receptors (PD-1/2B4/CD160) on HIV-1-specific CD8+ T-cells and higher viraemia, suggesting these represent a ‘hyper-exhausted’ cellular phenotype. However, HIV-2-specific CD8+ T-cell populations (largely from individuals with undetectable VLs and polyfunctional Gag responses) have surprisingly higher levels of PD-1/2B4/CD160 co-expression than bulk CD8+ populations (Fig 6a). Furthermore, a significant direct correlation was observed between co-expression of PD-1/2B4/CD160 and the
magnitude of the Gag-specific IFN-γ response (r = 0.48, p = 0.027, Fig S7). CMV-specific CD8+ T-cells displayed significantly lower co-expression of inhibitory receptors (Fig 6b and 6c). We therefore hypothesized that PD-1/2B4/CD160 expression may be related to cellular differentiation status. Accordingly, a strong correlation was observed between both PD-1 expression, as well as PD1/2B4/CD160 co-expression, with CD27+CD45RO+CD8+ T-cells (Fig S8) (r = 0.69, p = 0.0002 and r = 0.75, p < 0.0001 respectively).

**HIV-2-specific CD8+ T-cells display low levels of activation and proliferation ex vivo.**

Generalized cellular immune activation correlates with both VL and disease progression in HIV-1 and HIV-2, but activation and proliferation of HIV-2-specific CD8+ T-cells is less well characterized. CD38+Ki67+ co-expression was low on HIV-2-specific CD8+ T-cells and not significantly higher when compared to intra-patient bulk CD8+ T-cells (Median % (IQR) 2.49 (1.47, 3.5) vs. 1.71 (1.33, 2.33), p = 0.194, two-tailed Wilcoxon matched test, Fig S9a). Interestingly, a significant inverse correlation was observed between CD38 expression on HIV-2-specific T-cells and the % CD8+ IFN-γ response following Gag pool stimulation (r_s = -0.5104, p = 0.01, Fig S9b). A similar relationship was not observed between CD38 expression on bulk CD8+ T-cells and the IFN-γ response (r_s = -0.2176, p = 0.235, Fig S9c), suggesting activation of HIV-2-specific CD8+ T-cells per se may have a detrimental effect on function. Previous work has proposed that an increased susceptibility to apoptosis in CD8+ T-cells expressing high levels of CD38 may result in a failure to control HIV-1 replication. Accordingly, a strong inverse correlation was seen between CD38 expression and presence of Bcl-2, an anti-apoptotic marker, on HIV-2-specific CD8+ T-cells (r_s = -0.618, p = 0.001, Fig S9d).
DISCUSSION

A key factor in designing an effective T-cell vaccine for HIV-1 infection is the clear identification of immune correlates associated with viraemic control. Strong class I HLA associations and the association of a broad Gag-specific T-cell response with lower VLs implicate CD8+ T-cells as a crucial element of HIV-1 control. Nevertheless, the failure of the Merck Ad5-HIV vaccine demonstrates that the goal of an effective anti-HIV-1 T-cell vaccine remains elusive. HIV-2 infection provides an alternative model of natural HIV control, yielding important insights into HIV pathogenesis and viral control in humans. The current study provides detailed information about the qualities of CD8+ T-cells associated with natural HIV-2 control.

Using IFN-γ ELISpots, we have previously highlighted the association between the magnitude of HIV-2 Gag-specific T-cell responses and undetectable VL. We now demonstrate that this correlation is largely due to the CD8+ response. CD8+ T-cell polyfunctionality is associated with HIV-2 control, a feature shared with HIV-1-specific CD8+ T-cells from HIV-1 controllers. The quality (but not magnitude) of the CD4+ T-cell response may, however, be important: HIV-2 controllers had a greater proportion of polyfunctional CD4+ T-cells in the Gag-responsive population than viraemic subjects. The contribution of CD4+ help by secretion of cytokines such as IL-21 was also not assessed and may play a role. The importance of immunodominant Gag-specific responses in HIV-1 control has also been shown, but requires large studies to demonstrate a significant association. The hierarchical dominance of Gag-specific responses in HIV-2 is, however, more profound. Responses against HIV-2 Env (the second most commonly targeted HIV-2 protein) did not define viral control and further highlighted the greater polyfunctionality of Gag-specific responses in HIV-2 controllers. Our data also suggests that an HLA-B*3501-restricted HIV-2 p26 epitope (NPVPVGN) is commonly targeted and may contribute to viral control. Interestingly, HLA-B*3501 is associated with higher HIV-1 VLs and more rapid disease...
progression in HIV-1 clade-B infection\textsuperscript{28}, but protective outcomes in clade-C infection\textsuperscript{35}. The difference appears to be the greater Gag-directed response in clade-C-infected HLA-B*3501-positive subjects (specifically targeting the p24 epitope NPPIPVGDIY)\textsuperscript{36}, a situation seemingly paralleled in HIV-2 infection.

Maintaining a substantial breadth of T-cell responses is considered important in HIV-1 control and is associated with greater polyfunctionality and virus inhibition\textsuperscript{37}. This requirement for broad host responses, as a result of HIV-1 diversity and adaptation, poses a significant challenge for HIV-1 vaccine design. Our data suggest a stark contrast in HIV-2 infection, where CD8+ T-cells responding to a single HIV-2 epitope can be responsible for almost the entire Gag-specific response in a particular subject. Furthermore, whereas the persistence of high levels of tetramer-staining CD8+ T-cells is frequently observed in HIV-1 infection despite high viraemia\textsuperscript{38}, immunodominant Gag-specific CD8+ T-cell populations are often not detectable in viraemic HIV-2 subjects.

Different chronic viral infections are characterized by virus specific CD8+ T-cells displaying a particular cellular phenotype\textsuperscript{21}, and, accordingly, their effector functions vary. Several studies demonstrate that rare HIV-1 controllers possess HIV-1-specific CD8+ T-cells that are highly differentiated, with downregulation of markers such as CD27 and CD45RO\textsuperscript{19,39,40}, whereas others have shown no difference in cellular phenotype between controllers and progressors\textsuperscript{41}. Highly differentiated CD8+ T-cell subsets described in some HIV-1 controllers also have the ability to rapidly upregulate cytotoxic granule contents on stimulation, and express higher levels of the transcription factor T-bet\textsuperscript{19,20,42}. Surprisingly, we find that HIV-2-specific CD8+ T-cells (largely from individuals with low/undetectable VLs) are characterized by low levels of pre-loaded cytotoxic molecules, and that rapid upregulation of perforin after Gag stimulation does not distinguish HIV-2 controllers from viraemic subjects. In keeping with this observation, HIV-2-
specific CD8+ T-cells are of an earlier differentiated phenotype. Interestingly, cell supernatant alone from HIV-2 specific CD8+ T-cell clone cultures are able to partially suppress HIV-2 replication in CD4+ T-cell lines (AL, unpublished data). Further functional studies evaluating both the cytolytic activity and suppressive capacity of soluble factors secreted from primary HIV-2 specific CD8+ T-cells are required.

Studies exploring the relationship between antigen burden and cellular differentiation offer a potential explanation for the earlier differentiation phenotype of HIV-2 specific CD8+ T-cells43,44. Using both ex vivo antigen-specific CD8+ T-cell analysis and a model of in vitro T-cell priming, our previous study suggests that higher viral burden drives T-cell differentiation in HIV infection through greater immune activation43. Consistent with these data, HIV-1 sero-converters with higher VL set points have greater frequencies of differentiated effector memory HIV-1-specific CD8+ T-cells and higher activation levels44. Although studies of acute HIV-2 infection are lacking, it is likely that the VL set point of HIV-2 controllers is low, which may explain the persistent CD27 expression observed in chronic infection. The low activation levels observed in HIV-2-specific CD8+ T-cells in our current study are also in keeping with this hypothesis. Furthermore, we find high co-expression of PD-1/2B4/CD160 on HIV-2-specific CD8+ T-cells. Although robust data exist to suggest an inhibitory role for these markers in HIV-1 disease23, our findings support previous work suggesting PD-1 upregulation may also reflect changes in cellular differentiation45,46 and does not affect the ability of CD8+ T-cells to secrete cytokines in healthy adults46.

How could HIV-2-specific CD8+ responses with a limited specificity control HIV-2 for decades? Highly functional but narrow responses remain beneficial only if viral escape does not occur, resulting in uncontrolled viral replication in the absence of responses to other regions. Despite the fitness costs associated with mutation, escape from Gag-specific CD8+ T-cell responses is frequently seen in HIV-1 infection. Selection analyses comparing HIV-1 and HIV-2 env47 and gag
(TdS, unpublished) reveal that HIV-2 is under greater negative selective pressure than HIV-1, suggesting far greater functional and structural constraints are imposed on HIV-2. CD8+ T-cell epitope escape in HIV-2 has not been described and viral sequences from individuals with potent Gag-specific responses (and therefore selective pressure) show a high degree of conservation in respective epitopes48. Thus low VL set points and a functionally constrained virus may allow the persistence of initial CD8+ T-cell responses targeting conserved epitopes, which can contribute to viral control for years.

Our study is relatively limited by being based in a single cohort, although the long follow up in a genetically homogeneous population infected with the same HIV-2 group (A) is also a major strength that is difficult to reproduce in HIV-1 controller cohorts. It would be important to corroborate our findings in subjects with different HLA allele distributions. A key limitation worth discussing is the difficulty in determining whether the potent responses observed represent the cause of HIV-2 viral control, or if they simply reflect an undamaged immune system due to limited viral replication secondary to other factors. We fail to show an association between CD4+ counts (a marker of immunodeficiency) and the HIV-2 Gag specific CD8+ T-cell response, which may argue for a causative role. Alternative strategies to explore this problem may be helpful. Studying the evolution of HIV-2-specific CD8+ responses and VLs in HIV-2 controllers who acquire HIV-1 superinfection (i.e. suffer further immunological damage) may provide some insight. Such studies would also be key in determining whether T-cell responses, such as those described in this study, are responsible for attenuating the course of acute HIV-1 in HIV-1/2 dually infected subjects6 via cross-recognition of HIV-1 epitopes. The strongest evidence for a causative role could be established by achieving control of HIV-2 in viraemic individuals via immuno-therapeutic strategies designed to induce CD8+ T-cell responses. Such measures could provide proof-of-concept for future induction of HIV-1 immune-mediated control and allow valuable lessons to be
learned. It may also provide a therapeutic option for clinical care of HIV-2-infected subjects, who are often disadvantaged by sub-optimal ART regimes, compounded by the lack of reliable drug procurement in resource-limited settings.

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AUTHORSHIP CONTRIBUTIONS
TdS designed the study, undertook subject recruitment, performed experiments, data analysis and wrote the manuscript. YP designed and performed experiments and wrote the manuscript. AL performed data analysis and wrote the manuscript. IZ designed the experiments and wrote the manuscript. LL and HG undertook patient recruitment, processed samples and wrote the manuscript. MEB designed the experiments and wrote the manuscript. TV and MS designed the study, undertook subject recruitment and wrote the manuscript. LMY performed experiments and wrote the manuscript. CvT designed the study, undertook subject recruitment and wrote the manuscript. PE undertook subject recruitment, helped with data interpretation and wrote the
manuscript. AJ helped design the study and interpret the data and wrote the manuscript. HW helped design the study and interpret the data and wrote the manuscript. TD designed experiments, helped with data analysis and interpretation and wrote the manuscript. SRJ designed the study and experiments, undertook data interpretation and wrote the manuscript.

CONFLICTS OF INTEREST

We have no conflicts of interest to declare.

REFERENCES


Table 1. Characteristics of HIV-2 infected study subjects with undetectable (<100 copies/ml) and detectable plasma viral loads.

<table>
<thead>
<tr>
<th></th>
<th>Viral load &lt; 100 (n = 33)</th>
<th>Viral load ≥ 100 (n = 27)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age in years (range)</td>
<td>62.3 (31 – 89)</td>
<td>62.7 (36 – 91)</td>
<td>NS</td>
</tr>
<tr>
<td>Female</td>
<td>79%</td>
<td>74%</td>
<td>NS</td>
</tr>
<tr>
<td>CD4 absolute count (cells/µl) Median (IQR)</td>
<td>596 (468 – 856)</td>
<td>400 (252 – 530)</td>
<td>P = 0.004</td>
</tr>
<tr>
<td>CD4% Median (IQR)</td>
<td>37.9 (30.7 – 43.9)</td>
<td>28.4 (19.8 – 38.2)</td>
<td>P = 0.005</td>
</tr>
<tr>
<td>Medial Viral Load copies/ml (IQR)</td>
<td>&lt; 100</td>
<td>892 (693 – 20956)</td>
<td>NA</td>
</tr>
<tr>
<td>Follow up 13 – 21 years</td>
<td>27/33 (85%)</td>
<td>16/27 (59%)</td>
<td>P = 0.026</td>
</tr>
</tbody>
</table>

IQR = Inter-quartile range, NS = not significant, NA = not applicable
Figure 1. Comparison of HIV-2 Gag-specific (a) CD8+ and (b) CD4+ IFN-γ responses in HIV-2 controllers (n = 33) and viraemic subjects (n = 27) (two-tailed Mann-Whitney U test). Comparison of polyfunctional (3 or 4 function) Gag-specific CD4+ T-cell responses in HIV-controllers (n = 33) and viraemic subjects (n = 27), displayed as (c) % of polyfunctional Gag-specific CD4+ T-cells and (d) Proportion of the total Gag-specific CD4+ response that is polyfunctional (two-tailed Mann-Whitney U test). Bars represent medians. NS = not significant.
Figure 2. HIV-2 viral control is associated with polyfunctionality of the Gag-specific CD8+ T-cell response. (a) Proportion of response made up by different factors in HIV-2 controllers (Viral Load (VL) <100) and viraemic subjects (VL ≥ 100) (b) Magnitude of polyfunctional Gag-specific CD8+ T-cells in HIV-2 controllers and viraemic subjects (two-tailed Mann-Whitney U test) (c) Inverse correlation between CD8+ polyfunctionality and HIV-2 viral load (d) Direct correlation between CD8+ monofunctionality and HIV-2 viral load (Spearman’s rank correlation coefficient). All comparisons included n = 33 HIV-2 controllers and n = 27 viraemic subjects. NS = not significant.
Figure 3. HIV-2 Gag-specific CD8+ T-cells enumerated by peptide-MHC class I tetramers reveal a highly focused response. (a) Example of tetramer staining (b) A greater proportion of HIV-2 controllers (16/19) have detectable immunodominant Gag-specific tetramer populations than viraemic individuals (5/12) (p = 0.02) (c) A significant correlation exists between tetramer positive cells and the IFN-γ response to the total Gag pool (n = 31, viraemic donors in red), Spearman’s rank correlation coefficient. Where two different tetramer responses were present (in four subjects), these are added together. (d) The total Gag-specific IFN-γ response (as measured by ELISpot assays) according to presence or absence of responses to the four immunodominant epitopes (n = 64, data reanalyzed from Leligdowicz et al.11), two-tailed Mann-Whitney U test.
Figure 4. Perforin upregulation does not define HIV-2 control and high cytotoxic potential is not a feature of HIV-2 specific CD8+ T-cells. (a) CD8+ T-cells co-expressing perforin and IFN-γ are detectable following SEB stimulation, with varying proportions observed following HIV-2 Gag stimulation (example plots) (b) No difference between HIV-2 controllers (n = 33) and viraemic subjects (n = 27) in the degree of perforin co-expression (with either IFN-γ, TNF-α, IL-2 or CD107a) following stimulation with overlapping Gag peptides (two-tailed Mann Whitney U test). Displayed are medians and upper IQR (c) Comparison of cytolytic markers on bulk CD8+ and HIV-2 Gag-specific CD8+ T-cells (n = 25 tetramer-positive populations from 21 HIV-2 subjects: 16 controllers and 5 viraemic donors), two tailed Wilcoxon matched pairs test. Values from viraemic donors are highlighted in red (d) Comparison of cytolytic markers on different virus-specific CD8+ T-cells (Kruskal-Wallis test with Dunn’s post test for multiple comparisons). Bars represent medians. ** p < 0.01 * p < 0.05, NS = not significant.
Figure 5. HIV-2 specific CD8+ T-cells display an earlier differentiated phenotype than CMV specific CD8+ T-cells. (a) CD27 and (b) CD27/CD45RO co-expression according to viral specificity. *** p < 0.001 ** p < 0.01 * p < 0.05 in a Kruskal-Wallis test with Dunn’s post test for multiple comparisons. HIV-2 specific CD8+ populations (n = 25) are derived from 21 HIV-2 subjects (n = 16 controllers, n = 5 viraemic donors). CD27% expression on cells from viraemic donors is highlighted in red in figure 5(a). (c) Examples of CD27/CD45RO distribution of HIV-2, CMV and EBV specific CD8+ T-cells, with % distribution in each quadrant displayed.
Figure 6. HIV-2 specific CD8+ T-cells show high levels of PD-1, 2B4 and CD160 co-expression despite low viral loads (a) Higher expression of inhibitory receptors on HIV-2 specific CD8+ T-cells when compared to intra-patient bulk CD8+ populations (n = 25 tetramer-positive populations from 21 HIV-2 subjects: 16 controllers and 5 viraemic donors), two tailed Wilcoxon matched pairs test. Values from viraemic donors are highlighted in red (b) Comparison of PD-1+2B4+CD160+ co-expression on different virus-specific CD8+ T-cells, showing significantly lower co-expression on CMV-specific CD8+ T-cells when compared to HIV-2-specific or EBV-specific CD8+ T-cells * p < 0.05 in a Kruskal-Wallis test with Dunn’s post test for multiple comparisons. Values from viraemic donors are highlighted in red (c) Example of inhibitory receptor expression on total CD8+, HIV-2 specific and CMV specific CD8+ T-cell from a single donor. Red dots represent cells co-expressing CD160 in addition to PD-1 and/or 2B4. % distribution in each quadrant is displayed (PD-1 vs 2B4), with the boxed number denoting % of cells co-expressing all three markers.
Correlates of T-cell mediated viral control and phenotype of CD8+ T-cells in HIV-2, a naturally contained human retroviral infection