Table 1. Clinical Characteristics of Patients With and Without CMV-GID

	CMV-GID (n=12)	*Non CMV-GID (n=88)*	p *Value*
Age (IQR)	39 (36–46)	40 (37–51)	0.451
Male gender (%)	11 (91.7)	87 (98.9)	0.227
CD4 count (/μL) (IQR)	68.5 (28.8–123.3)	84 (38.3–151.0)	0.356
HIV viral load (log10/mL) (IQR)	4.58 (3.27–5.24)	2.84 (1.60–5.08)	0.084
History of HARRT (%)	3 (5.2)	55 (62.5)	0.016
MSM (%)	9 (75.0)	69 (78.4)	0.723
Positive CMV antigenemia (%)	9 (75.0)	18 (20.5)	<0.001
Epigastric pain (SD)	2.5 (2.1)	1.8 (1.3)	0.373
Heartburn (SD)	2.5 (1.5)	1.8 (1.3)	0.064
Nausea and vomiting (SD)	2.4 (1.7)	2.0 (1.5)	0.384
Odynophagia (SD)	2.1 (1.7)	1.7 (1.5)	0.481
Chronic diarrhea (SD)	2.3 (1.3)	1.8 (1.4)	0.078
Bloody stool (SD)	2.5 (2.0)	1.7 (1.5)	0.021

CD4 cell counts within 1 week and HIV-RNA viral load within 1 month were checked at the day of endoscopy. A positive result for real-time HIV RNA was defined as ≥40 copies/mL. History of HAART was collected from the medical records prior to endoscopy. Sexual behavior was defined as men who have sex with men (MSM) or heterosexual.

CMV, cytomegalovirus; GID, gastrointestinal disease; HAART, highly active antiretroviral therapy; IQR, interquartile range; MSM, men who have sex with men; SD, standard deviation.

study.[8–10,22–26] This difference could be explained by the fact that the current study focused on gastrointestinal disease, while previous studies included various CMV diseases such as retinitis, cholangitis, pneumonia, and encephalitis.[8–10,22–26] The diagnostic accuracy of CMV antigenemia may vary depending on the site and extent of organ/tissue involvement.

Identification of CMV cells in tissue samples obtained by endoscopic biopsy is considered the gold standard for the diagnosis of CMV-GID.[1,2,6] The endoscopic findings in CMV-GID include ulcer and mucosal inflammation;[16,17] however, physicians may not consider it necessary to take a biopsy in patients with only mucosal inflammation without ulceration. Even in cases of severe deep or bleeding ulcers, some physicians may hesitate to perform a biopsy. In such cases, no definite diagnosis of CMV-GID can be made. Our results suggest that the CMV antigenemia assay is to some extent useful for the diagnosis of CMV-GID in patients with endoscopic findings, especially when CMV positive cell counts are high. Considering the high specificity and high positive LR (5.5) of the positive CMV cell count ≥5, the use of this method before endoscopy could potentially avoid complications due to biopsy.

One limitation of this study was the single-center nature of the investigation. Significant differences in independent factors were not detected in the present study probably due to the small number of patients with CMV-GID. For example, we used gastrointestinal symptoms with score of 7 points on the Likert scale, but the differences in most symptoms between patients with or without CMV-GID did not reach statistical significance due to the small number of cases. Further studies based on larger population are needed. Another limitation is a selection bias related to the selection criteria applied in the present study: only patients who underwent endoscopy for such reasons as symptoms and screening were included in the study.

In conclusion, the CMV antigenemia assay showed relatively good sensitivity and specificity for the diagnosis of CMV-GID in patients with HIV infection. Furthermore, specificity and positive LR improved when the cutoff value of CMV cell count was increased from 1 to ≥5 positive cells per 300,000 granulocytes. Considering the high specificity of the test, the use of this method before endoscopy could potentially avoid complications due to biopsy.

Table 2. Diagnostic Accuracy of CMV Antigenemia Assay for CMV-GID Using Different Cutoff Values and History of HAART

	Sensitivity (95%CI)	*Specificity (95%CI)*	*PPV (95%CI)*	*NPV (95%CI)*	*LR+ (95%CI)*	*LR− (95%CI)*	*OR (95%CI)*
CMV antigenemia ≥1 positive cell	75.0% (42.8–94.5)	79.5% (69.6–87.4)	33.3% (16.5–54.0)	95.9% (8.5–99.1)	3.7 (2.2–6.2)	0.31 (0.11–0.84)	11.7 (3.1–44)
CMV antigenemia ≥5 positive cells	50.0% (21.1–78.9)	90.9% (82.9–96.0)	42.9% (17.7–71.1)	93% (85.4–97.4)	5.5 (2.3–13.1)	0.55 (0.31–0.97)	10.0 (2.7–37.1)
History of HAART							
Yes[a]	66.7% (9.4–99.2)	83.6% (71.2–92.2)	18.2% (2.3–51.8)	97.9% (88.7–99.9)	3.7 (2.2–6.2)	0.31 (0.11–0.84)	10.2 (1.2–NA)
No[a]	77.8% (40.0–97.2)	72.7% (54.5–86.7)	43.8% (19.8–70.1)	92.3% (74.9–99.1)	2.9 (1.5–5.5)	0.31 (0.88–1.1)	9.33 (1.79–NA)

[a]Cutoff value of ≥1 positive cell per 300,000 granulocytes was used in the analysis.

CMV, cytomegalovirus; HAART, highly active antiretroviral therapy; LR+, positive likelihood ratio; LR−, negative likelihood ratio; NPV, negative predictive value; OR, odds ratio; PPV, positive predictive value.

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Author Disclosure Statement

The other authors declare no conflict of interest.

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CD8$^+$ T Cell Cross-Reactivity Profiles and HIV-1 Immune Escape towards an HLA-B35-Restricted Immunodominant Nef Epitope

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Abstract

Antigen cross-reactivity is an inbuilt feature of the T cell compartment. However, little is known about the flexibility of T cell recognition in the context of genetically variable pathogens such as HIV-1. In this study, we used a combinatorial library containing 24 billion octamer peptides to characterize the cross-reactivity profiles of CD8$^+$ T cells specific for the immunodominant HIV-1 subtype B Nef epitope VY8 (VPLRPMTY) presented by HLA-B*35:01. In conjunction, we examined naturally occurring antigenic variations within the VY8 epitope. Sequence analysis of plasma viral RNA isolated from 336 HIV-1-infected individuals revealed variability at position (P) 3 and P8 of VY8; Phe at P8, but not Val at P3, was identified as an HLA-B*35:01-associated polymorphism. VY8-specific T cells generated from several different HIV-1-infected patients showed unique and clonotype-dependent cross-reactivity footprints. Nonetheless, all T cells recognized both the index Leu and mutant Val at P3 equally well. In contrast, competitive titration assays revealed that the Tyr to Phe substitution at P8 reduced T cell recognition by 50–130 fold despite intact peptide binding to HLA-B*35:01. These findings explain the preferential selection of Phe at the C-terminus of VY8 in *HLA-B*35:01*$^+$ individuals and demonstrate that HIV-1 can exploit the limitations of T cell recognition *in vivo*.

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Introduction

Hypervariable viruses such as HIV-1 can escape from human leukocyte antigen class I (HLA-I)-restricted CD8$^+$ T cell responses by acquiring viral genomic mutations within or near immunogenic epitopes. Such immune escape pathways can be extremely reproducible and broadly predictable based on host HLA-I alleles at a population level [1,2]. Somewhat paradoxically, however, antigen cross-reactivity is an inbuilt feature of the T cell compartment [3,4]. Indeed, a single autoimmune T cell receptor (TCR) has recently been shown to recognize more than a million different peptides within a broad cross-reactivity profile encompassing unrelated amino acid substitutions [5]. Furthermore, several lines of evidence suggest that certain CD8$^+$ T cell subsets with the capacity to cross-recognize naturally occurring viral variants are advantageous for viral control *in vivo* [6–11]. However, the true extent of HIV-1-specific T cell cross-reactivity remains elusive. In the present study, we characterized the cross-reactivity footprints of HIV-1-specific CD8$^+$ T cells using combinatorial peptide library (CPL) scanning to cover all possible amino acid variations at each position of an octamer epitope. Additionally, we analyzed antigenic variation within the targeted epitope region of HIV-1 subtype B. Our investigations focused on CD8$^+$ T cell responses specific for the immunodominant HIV-1 Nef epitope VY8 (VPLRPMTY) presented by HLA-B*35:01 [12,13].

Materials and Methods

Ethics Statement

All study participants provided informed, written consent at the AIDS Clinical Center, National Center for Global Health and Medicine, Japan. The study was approved by the Institutional Review Board of Kumamoto University and National Center for Global Health and Medicine.

Sequence Analysis of Autologous HIV-1

Treatment-naïve individuals (n = 336) with chronic HIV-1 infection (>90% subtype B) attending the AIDS Clinical Center (International Medical Center of Japan) were enrolled for autologous HIV-1 sequence analysis. The median [IQR] plasma viral load was 95,000 [31,000–350,000] copies/ml; the median

Table 1. TCR β composition of $CD8^+$ T cell lines.

Patient	β chain			
	V gene	J gene	CDR3 sequence	Frequency
Pt-100	BV2*01	BJ2-7*01	CASSGEGNYEQYF	1/31
			CASTTDRVYEQYF	1/31
	BV3-1*01	BJ2-5*01	CASSTSSVTETQYF	2/31
		BJ2-7*01	CASSQDIAGVHEQYF	1/31
	BV4-1*01	BJ2-1*01	CASSQTSGSYNEQFF	1/31
	BV6-1*01	BJ1-5*01	CASSEASGIYEQYF	1/31
		BJ2-7*01	CASSEASGIYEQYF	1/31
	BV10-1*01	BJ2-1*01	CASSAAGVEYNEQFF	1/31
	BV11-2*01	BJ1-1*01	CASSFDIVNTEAFF	1/31
		BJ2-1*01	CASSPDLVDNEQFF	4/31
		BJ2-5*01	CASSGAWTGGGETQYF	2/31
		BJ2-7*01	CASSLDLVSYEQYF	1/31
			CASSLGIGRAYEQYF	1/31
	BV12-3*01	BJ1-4*01	CASSLRFATNEKLFF	1/31
	BV27*01	BJ2-5*01	CASSFDTNQETQYF	1/31
		BJ2-7*01	CASSLDTNGYEQYF	1/31
			CASSFQLAGVHGQYF	1/31
			CASSPRLDDEQYF	2/31
			CASSLDTSGYEQYF	2/31
			CASSSDREDSHEQYF	2/31
	BV28*01	BJ2-2*01	CASSSTDRAIPNTGELFF	1/31
		BJ2-3*01	CASSLPGLDSTDTQYF	1/31
		BJ2-7*01	CASSEGQGRYEQYF	1/31
Pt-168	BV2*01	BJ2-7*01	CASSESLAGGPYEQYF	7/31
	BV3-1*01	BJ2-3*01	CASSQEGADTQYF	2/31
	BV3-1*02	BJ2-3*01	CASSQEGAGTQYF	1/31
	BV6-2*01	BJ1-1*01	CASSGGRTDENTEAFF	1/31
		BJ2-1*01	CASSYEREDSGNEQFF	1/31
	BV11-2*01	BJ2-7*01	CASSLDVAGSYEQYF	1/31
			CASSLDIVSYEQYF	1/31
	BV11-3*03	BJ2-3*01	CASSLVLGTGTDTQYF	1/31
	BV12-3*01	BJ2-3*01	CASSWDSISTDTQYF	1/31
		BJ2-7*01	CASSSDGYEQYF	3/31
	BV12-5*01	BJ2-2*01	CASGLAMVVSGELFF	1/31
	BV15*02	BJ2-1*01	CATSRDLVEDEQFF	2/31
	BV20-1*05	BJ2-2*01	CSARDPRTDRGNTGELFF	1/31
	BV24-1*01	BJ2-3*01	CATSVRDDLTGNGPDTQYF	2/31
	BV27*01	BJ2-3*01	CASSLDLRPDTQYF	1/31
	BV28*01	BJ2-5*01	CASSLLGEETRETQYF	4/31
	BV30*01	BJ2-5*01	CAWHTVRVQETQYF	1/31

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[IQR] $CD4^+$ T cell count was 242 [64.5–367.5] cells/mm^3. We determined autologous *nef* sequences from plasma viral RNA using a previously reported direct sequencing method [13].

Table 2. TCR β composition of $CD8^+$ T cell clones.

Patient	Clone	β chain		
		V gene	J gene	CDR3 sequence
Pt-19	19-136	BV7-2*03	BJ2-1*01	CASSPTPQGDYEQFF
	19-139	BV11-2*01	BJ1-1*01	CASSLDLVSTEAFF
Pt-33	33-S1	BV4-2*01	BJ2-3*01	CASSQAADAAITDADTQYF
Pt-100	100-K51	BV27*01	BJ2-5*01	CASSFDTNQETQYF
	100-K105	BV11-2*01	BJ1-1*01	CASSFDIVNTEAFF
	100-K810	BV27*01	BJ2-7*01	CASSFQLAGVHGQYF

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Generation and Maintenance of $CD8^+$ T cell Lines and Clones

The $CD8^+$ T cell clones (19–136, 19–139 and 33-S1) were established previously [13]. Additional $CD8^+$ T cell lines and clones were generated by VY8 peptide stimulation of peripheral blood mononuclear cells (PBMCs) isolated from *HLA-B*35:01*$^+$ individuals with chronic HIV-1 infection (Pt-100 and Pt-168) with 10 nM of VY8 (VPLRPMTY) peptide. The Institutional Review Board of the National Center for Global Health and Medicine approved both taking samples and generating cell lines, and patients provided the written informed consent. All $CD8^+$ T cell lines and clones were maintained in RPMI 1640 supplemented with 10% fetal calf serum, 10 IU recombinant human interleukin (IL)-2, antibiotics and L-glutamine.

Analysis of TCR-encoding Genes

TCR-encoding genes of $CD8^+$ T cell lines and clones were obtained by using a SMART PCR cDNA synthesis kit (Clontech) and analyzed with reference to the ImMunoGeneTics database (http://imgt.cines.fr) as described previously [14].

T cell Sensitivity Assay

Secretion of cytokines and chemokines by virus-specific $CD8^+$ T cells in response to specific antigen provides a useful tool for quantitative assessment of antigen recognition [15,16]. MIP-1β was used as a functional readout in this study since it is one of the most sensitive means to assess functional avidity of human $CD8^+$ T cells as previously described [15–17]. Briefly, 3×10^4 T cells were mixed with 6×10^4 HLA-B*35:01-expressing C1R cells (C1R-B3501), either unpulsed or pulsed with cognate peptide across a range of concentrations. After overnight incubation at 37°C, the supernatant was harvested and assayed for MIP-1β content by ELISA as described previously [5,17]. The amount of MIP-1β released in the absence of the peptide was subtracted as background. It should be noted that the VY8 peptide titration experiments of T cell clones 136 and 139 exhibited comparable results when IFN-γ [13] and MIP-1β were used as readouts (data not shown).

Figure 1. Amino acid residues preferentially recognized by VY8-specific CD8$^+$ T cells. Graphical representation showing relative preference for amino acid residues recognized by VY8-specific T cell lines and clones based on the CPL scan data shown in Figure S1. Responses >20% were included. A web-based application, WebLogo 3 (http://weblogo.threeplusone.com/), was used to generate the graphic. Colours represent physicochemical properties: polar (G, S, T, Y and C), green; neutral (Q and N), purple; basic (K, R and H), blue; acidic (D and E), red; hydrophobic (A, V, L, I, P, W, F and M), black. The index residues at each position are outlined in yellow. Residue size is proportional to T cell recognition preference.
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Octamer Combinatorial Peptide Library (CPL) Scan

The octamer CPL contained a total of 2.4×10^{10} different peptides (PepScan) divided into 160 sub-mixtures in positional scanning format as described previously [4,18]. Target C1R-B3501 cells (6×10^4 cells/well) were pre-incubated in the absence or presence of CPL sub-mixtures (100 μg/ml). Effector T cells (3 x 10^4 cells/well) were then added and incubated overnight at 37°C. Supernatant was collected and analyzed for MIP-1β content by ELISA as described previously [5,17]. Background-subtracted results were expressed as % response, normalized with respect to the VY8 index residue. A response >20% was considered positive.

Results and Discussion

Clonotypic Characterization of VY8-specific T cells

CD8$^+$ T cell lines were established from two *HLA-B*35:01*$^+$ individuals with chronic HIV-1 infection (Pt-100 and Pt-168). Analysis of TCR β usage by these T cell lines revealed multiple clonotypes, with 23 and 17 distinct TCR β sequences for Pt-100 and Pt-168, respectively (Table 1). This observation is consistent with previous studies showing the oligoclonal nature of immunodominant HIV-1-specific CD8$^+$ T cell populations [19,20]. The CD8$^+$ T cell clones K51, K105 and K810 were generated from patient Pt-100 by limiting dilution of VY8-specific T cell lines. Monoclonality was confirmed by TCR β analysis and all three sequences were encompassed within the TCR repertoire of the parental T cell lines (Table 2). Additional CD8$^+$ T cell clones (136, 139, and S1) previously established from two separate *HLA-B*35:01*$^+$ HIV-1-infected individuals [12,13] showed distinct TCR β chain usage (Table 2) and were also used for cross-reactivity studies.

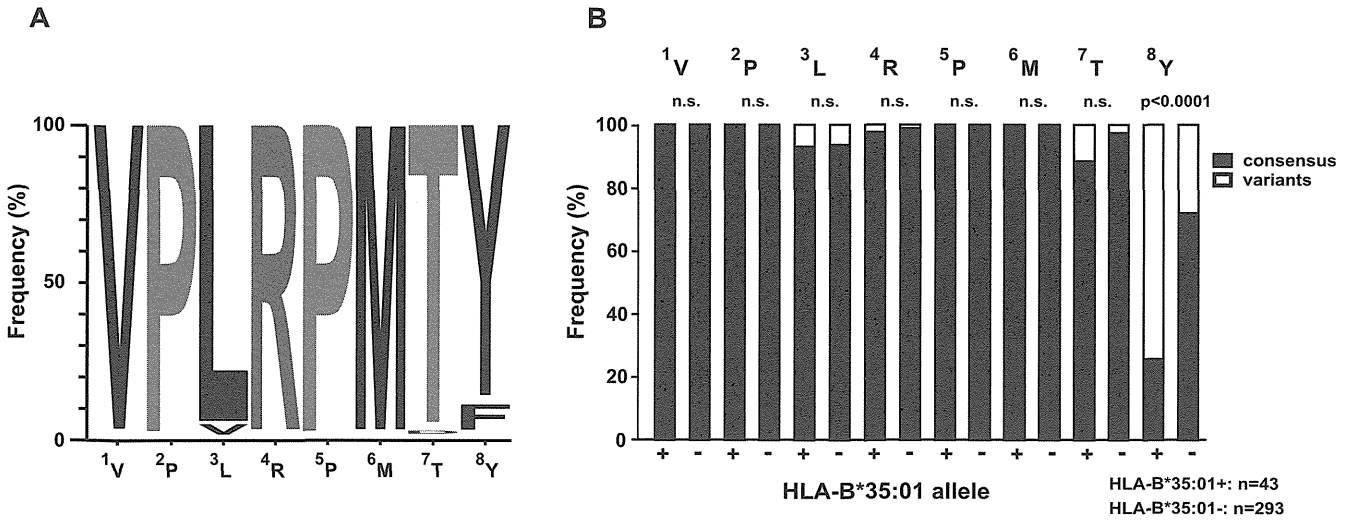

Figure 2. Naturally arising antigenic variations in the VY8 epitope. (**A**) Graphical representation showing the frequency of amino acid residues within the VY8 epitope in subtype B Nef sequences retrieved from the Los Alamos database (n = 1191). WebLogo 3 was used to generate the graphic. (**B**) The frequency of consensus (subtype B) and variant amino acid residues at each position of the VY8 epitope is shown for autologous plasma viral sequences derived from a total of 336 HIV-1-infected individuals, segregated according to *HLA-B*35:01* status. Statistical analysis was performed using Fisher's exact test. *n.s.*, not significant.
doi:10.1371/journal.pone.0066152.g002

Cross-reactivity Analysis of VY8-specific T cells

The cross-reactivity profiles of VY8-specific T cell lines and clones were analyzed using a CPL containing a total of 2.4×10^{10} different octamer peptides, which allowed qualitative mapping of preferred T cell recognition residues at each position along the peptide backbone [4,18]. Different VY8-specific T cell lines and clones preferentially recognized different amino acid residues across the octamer peptide backbone (Figure S1). We employed a graphical representation of these preferential recognition residues by the VY8-specific T cells (Figure 1). Despite these unique cross-reactivity patterns, all T cells tested recognized the index VY8 residues efficiently (Figure 1). This finding contrasts with previous observations using tumor-specific and autoreactive T cell clones [5,21–23], which typically prefer non-index amino acid residues. Across all clones, more stringent recognition was observed at position 2 (P2) and P8 (Figure 1). This most likely reflects the anchor role of these positions in peptide binding to HLA-B*35:01 [12,24]. The VY8-specific T cell clones, K51, K105 and K810, showed inherently unique cross-reactivity footprints but less flexible cross-recognition compared to the parental T cell line (Figure 1), suggesting increased coverage of viral antigenic variation through polyclonal TCR cross-reactivity.

Naturally Occurring Antigenic variations within the VY8 Epitope

To investigate the correlation between T cell cross-reactivity and naturally occurring antigenic variation, we analyzed sequence polymorphisms within the VY8 epitope. Despite the remarkable variability of HIV-1 Nef, VY8 is highly conserved, most likely due to its location partially within a Src homology 3 binding motif that

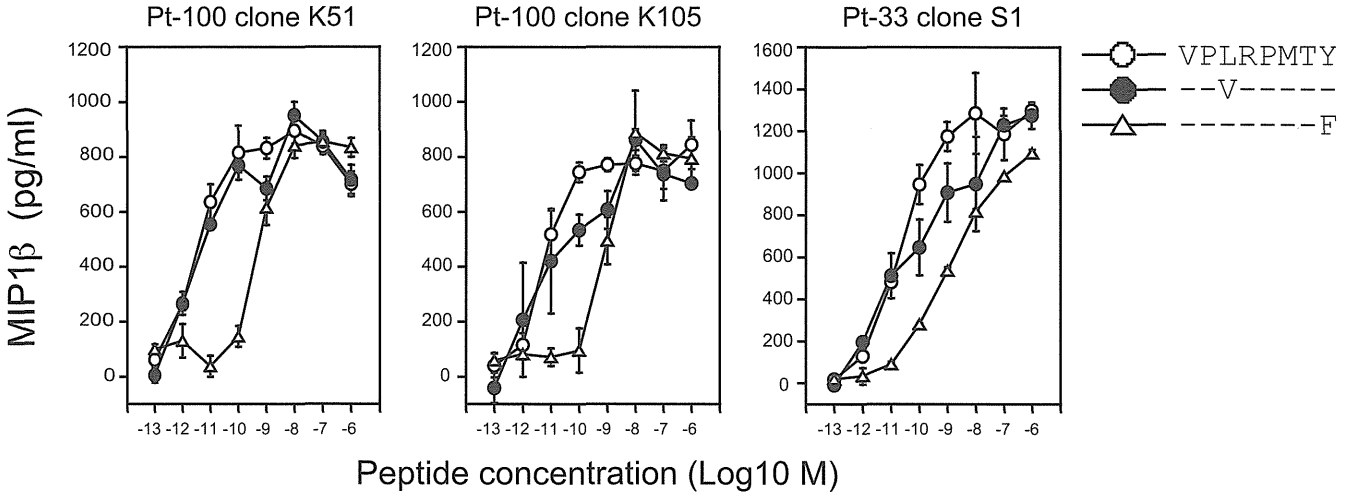

Figure 3. VY8-specific CD8$^+$ T cell sensitivity towards peptide variants. The sensitivity of T cell clones towards the VY8, VY8-3V and VY8-8F peptides was quantified by measuring the amount of MIP-1β secreted in response to antigen stimulation. Data are representative of duplicate assays and standard deviation from the mean of two replicates is shown.
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Table 3. Sensitivity of VY8-specific $CD8^+$ T cells.

$CD8^+$ T cells		EC_{50} (M)		
		VY8	VY8-3V	VY8-8F
lines	Pt-100	5.9×10^{-12} (x 1)	nd	3.9×10^{-10} (x 66)
	Pt-168	4.0×10^{-12} (x 1)	nd	4.3×10^{-10} (x 105)
clones	33-S1	2.3×10^{-11} (x 1)	3.9×10^{-12} (x 0.17)	1.2×10^{-9} (x 52)
	100-K51	3.1×10^{-12} (x 1)	5.8×10^{-12} (x 1.8)	4.2×10^{-10} (x 135)
	100-K105	5.1×10^{-12} (x 1)	3.9×10^{-12} (x 0.76)	6.7×10^{-10} (x 131)

EC_{50}, determined by duplicate assays; nd, not done; in parenthesis, fold changes in sensitivity relative to index.

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is critical for several Nef functions [25], including HLA-I down-regulation [13,26]. Nevertheless, in the Los Alamos HIV Sequence database (http://www.hiv.lanl.gov/content/index), some variability within HIV-1 subtype B has been reported at P3 Leu and P8 Tyr of the VY8 epitope, with 2.4% and 8.2% of viral clones showing polymorphisms in these positions, respectively (Figure 2A). Given that approximately 40% of Nef sequence polymorphisms are associated with host HLA-I alleles [1], we examined these particular variants for HLA-I association. Our previous smaller study of 69 HIV-1-infected patients indicated that Phe at P8 might be associated with the *HLA-B*35:01* allele [13]. To confirm this association and examine polymorphisms at P3, we recruited a larger cohort comprising 336 treatment-naïve individuals with chronic HIV-1 infection and determined autologous *nef* sequences from plasma viral RNA. Although we found some variability at P3 (3%), there were no statistically significant amino acid differences at P1–P7 between individuals with or without *HLA-B*35:01* (Figure 2B). In fact, CPL scanning showed that, at P3, hydrophobic residues including both the index Leu and mutant Val were preferentially recognized by all VY8-specific T cells tested (Figure 1). Such flexible TCR recognition at P3 helps to explain why the Val mutant is not selected in *HLA-B*35:01*$^+$ individuals. Conversely, we found a statistically significant difference in the frequency of polymorphisms at P8 between individuals with or without *HLA-B*35:01* (Figure 2B); indeed, the vast majority (74%) of *HLA-B*35:01*$^+$ donors harboured viral sequences with Phe at P8. However, CPL scanning showed that Phe was a favoured amino acid residue recognized by T cell lines and some clones, such as K105 (Figure 1 and Figure S1). In these instances, CPL data alone do not simply explain the emergence of this viral mutation in *HLA-B*35:01*$^+$ individuals.

VY8-specific T cell Sensitivity Towards Peptide Variants

To verify the effect of single mutations within the VY8 peptide on TCR sensitivity, we performed competitive titration assays across our panel of VY8-specific T cells (Figure 3). Consistent with the CPL scan data, all T cells tested recognized the VY8 and VY8-3V peptides comparably (<2 fold difference in EC_{50} values; Table 3). In contrast, the EC_{50} values for VY8-8F were >50 fold higher than index for all T cells tested (Table 3). These observations are consistent with previous reports showing that VY8-specific T cells could not recognize $CD4^+$ T cells or macrophages infected with HIV-1 carrying this Nef variant at P8 [13,26].

Although P8 is an anchor residue for VY8, our previous HLA-I stabilization studies showed comparable binding activity between HLA-B*35:01 and either VY8 or VY8-8F [13]. The crystal structure of the VY8/HLA-B*35:01 complex shows that P8 Tyr lies deep inside the F pocket of the HLA-I molecule [24]. Substitution at this position with the aromatic residue Phe may not induce substantial structural changes. Consequently, impaired T cell recognition of P8 Phe may be mediated by indirect conformational changes imposed by the peptide upon TCR binding [17]. In the context of HLA-A*02:01, however, a Tyr to Phe substitution at the secondary anchor P3 of an antigenic peptide (SLFNTVATL) leads to unexpectedly large conformational changes in the peptide backbone [27]. Accordingly, further structural studies are needed to elucidate the precise mechanism through which anchor residue substitution leads to impaired T cell recognition of the VY8 epitope.

Previous studies have shown that the double substitution of Arg-71 to Thr and Tyr-81 to Phe (P8 at VY8) [13], or Pro-75 to Ala (P2 at VY8) as a single mutation, impair Nef-mediated down-regulation of HLA-I and thereby increase the susceptibility of HIV-1-infected cells to killing by $CD8^+$ T cells targeting other epitopes [26,28]. In contrast, the Tyr-81 to Phe (P8 at VY8) mutation alone exerts virtually no effect on Nef-mediated activities [13,26]. Collectively, these data suggest that the P8 Phe mutation does not compromise viral fitness.

Concluding Remarks

$CD8^+$ T cell responses against the immunominant HIV-1 subtype B-derived Nef epitope VY8 presented by HLA-B*35:01 are highly polyclonal, broadly cross-reactive and capable of tolerating natural viral variation with one notable exception. Specifically, the observed Phe substitution at P8, which is neutral in terms of Nef-mediated function [13,26], was found to reduce $CD8^+$ T cell recognition by >50 fold. The association of this mutation with *HLA-B*35:01*$^+$ strongly suggests that evasion of VY8-specific $CD8^+$ T cell activity confers a selection advantage *in vivo*. Thus, even $CD8^+$ T cell responses with extensive cross-reactivity profiles can succumb to immune escape at a single position.

Supporting Information

Figure S1 CPL scanning of VY8-specific $CD8^+$ T cells. The cross-reactivity profiles of T cell lines and clones specific for VY8 were tested by using 160 CPL sub-mixtures (100 μg/ml) comprising a total of 2.4×10^{10} different octamer peptides. In every peptide mixture, one position has a fixed amino acid residue and all other positions are degenerate, with the possibility of any one of 19 natural amino acids being incorporated in each individual position (cysteine is excluded). The amount of MIP-1β secreted in response to antigen was quantified by ELISA. Data are background-subtracted and the relative T cell response is shown as a ratio of MIP-1β production with respect to the index residue at each position. Responses >20% were considered positive and used to construct Figure 1. A representative set of duplicate assays is shown. Red bars depict residues corresponding to the VY8 index sequence.
(EPS)

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Author Contributions

Conceived and designed the experiments: CM JJM AKS TU. Performed the experiments: CM JJM ZH SCM TU. Analyzed the data: CM JJM ZH

SCM DAP AKS TU. Contributed reagents/materials/analysis tools: HG SO. Wrote the paper: CM JJM DAP AKS TU.

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MAJOR ARTICLE HIV/AIDS

Naturally Selected Rilpivirine-Resistant HIV-1 Variants by Host Cellular Immunity

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Background. Rilpivirine is listed as an alternative key drug in current antiretroviral therapy (ART) guidelines. E138G/A/K in human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) are rilpivirine resistance-associated mutations and can be identified in a few ART-naive patients, although at low frequency. The 138th position in HIV-1 RT is located in one of the putative epitopes of human leukocyte antigen (HLA)–B*18–restricted cytotoxic T lymphocytes (CTLs). CTL-mediated immune pressure selects escape mutations within the CTL epitope. Here we tested whether E138G/A/K could be selected by HLA-B*18-restricted CTLs.

Methods. The amino acid variation at the 138th position was compared between ART-naive HIV-1–infected patients with and without HLA-B*18. The optimal epitope containing the 138th position was determined and the impact of E138G/A/K on CTL response was analyzed by epitope-specific CTLs. The effect of E138G/A/K on drug susceptibility was determined by constructing recombinant HIV-1 variants.

Results. The prevalence of E138G/A/K was 21% and 0.37% in 19 and 1088 patients with and without HLA-B*18, respectively (odds ratio, 72.3; $P = 4.9 \times 10^{-25}$). The CTL response was completely abolished by the substitution of E138G/A/K in the epitope peptide. E138G/A/K conferred 5.1-, 7.1-, and 2.7-fold resistance to rilpivirine, respectively.

Conclusions. E138G/A/K can be selected by HLA-B*18-restricted CTLs and confer significant rilpivirine resistance. We recommend drug resistance testing before the introduction of rilpivirine-based ART in HLA-B*18-positive patients.

Keywords. rilpivirine; E138G/A/K; HLA-B*18; CTL.

Rilpivirine is a new-generation nonnucleoside reverse transcriptase inhibitor (NNRTI), with noninferior clinical efficacy demonstrated in large clinical trials, compared with efavirenz [1, 2], and is listed as an alternative key drug in current antiretroviral therapy (ART) guidelines [3, 4]. In those clinical trials, rilpivirine showed more-favorable safety and tolerance profiles compared with efavirenz, although it was also associated with a higher virological failure rate. The most commonly observed NNRTI resistance-associated mutation in rilpivirine-treated patients with virological failure has so far been E138 K [1, 2]. Not only E138 K, but also other substitutions at the 138th position in human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT), might confer significant rilpivirine resistance [5–7]. The glutamic acid at the 138th position (E138) is well conserved among HIV-1 strains and clinical isolates throughout clades [8]. However, some ART-naive patients are infected with HIV-1 variants harboring other amino acids at the 138th position (E138X), although the proportion of such patients is low [9]. The 138th position is located in one of the putative epitopes of human leukocyte antigen (HLA)–B*18-restricted cytotoxic T lymphocytes (CTLs) [10, 11]. Because CTL immune pressure often selects escape mutations within the epitope [11], E138X may be selected by HLA-B*18-restricted CTLs. In this study, we analyzed the frequency of amino acid variations at the 138th position in ART-naive patients with or without

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Table 1. Amino Acid Variations at the 138th Position of HIV-1 Reverse Transcriptase and Human Leukocyte Antigen–B*18

Amino Acid	HLA-B*18(+)	HLA-B*18(−)
E138 (wild-type)	15	1084
E138G	2	1
E138A	1	2
E138K	1	1

Abbreviation: HLA, human leukocyte antigen.

HLA-B*18, determined the impact of E138X on CTL response, and analyzed the drug susceptibility of recombinant HIV-1 variants harboring E138X.

METHODS

Sequences of HIV-1 Reverse Transcriptase

HIV-1 RT sequences were analyzed using viral RNA extracted from plasma samples [12], and HLA type was determined by standard sequence-based genotyping in 1107 ART-naive infected individuals who visited the Outpatient Clinic of the AIDS Clinical Center, National Center for Global Health and Medicine, Tokyo, between 2003 and 2012. The amino acid variation at the 138th position of HIV-1 RT was compared between individuals with and those without HLA-B*18, and the statistical significance of the difference was analyzed by Fisher exact test using the Statistical Package for Social Sciences, version 17.0 (SPSS, Chicago, Illinois). This study was approved by the institutional ethical committee of the National Center for Global Health and Medicine, and written informed consent was obtained from all the participants according to the Declaration of Helsinki.

Intracellular Cytokine Staining Assay

HIV-1-derived peptides and mutant peptides were synthesized using an automated multiple peptide synthesizer and purified by high-performance liquid chromatography. Peripheral blood mononuclear cells (PBMCs) from chronically HIV-1–infected HLA-B*18-positive patients were stimulated with the peptide (100 nM) in culture medium (RPMI 1640 medium supplemented with 10% fetal calf serum and 200 U/mL recombinant human interleukin 2). After 14 days in culture, the cells were assessed for interferon (IFN)–γ production activity using a FACSCanto II (BD Biosciences, San Jose, California) [13, 14].

Drug Susceptibility Assay

The desired mutations were introduced into the *XmaI-NheI* region of pTZNX, which encodes the 15th–267th positions of HIV-1 RT (strain BH10) [15, 16]. The *XmaI-NheI* fragment was inserted into pNL_{H219Q}, which was modified from pNL101 and encoded the full genome of HIV-1. Each molecular clone was transfected into COS-7 cells, and the obtained virions were harvested 48 hours after transfection and stored at −80°C until use. Efavirenz and nevirapine were generously provided by Merck Co, Inc (Rahway, New Jersey) and Boehringer Ingelheim Pharmaceutics Inc (Ridgefield, Connecticut), respectively. Etravirine and rilpivirine were purchased from Toronto Research Chemicals Inc (North York, Ontario, Canada). The susceptibility of recombinant HIV-1 variants to efavirenz, nevirapine, etravirine, and rilpivirine was determined in triplicate and repeated 3 times [16]. Fold resistance was calculated by comparing the viral 50% inhibitory concentration (IC_{50}) with that of monoclonal wild-type HIV-1.

Structural Modeling

We constructed structural models of the HIV-1 RT and rilpivirine complex by computational analysis, as described in our

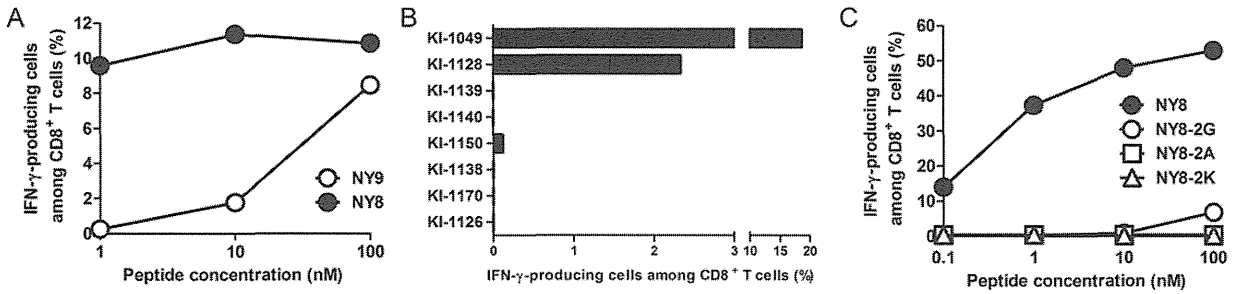

Figure 1. Recognition of human leukocyte antigen (HLA)–B*18-restricted $CD8^+$ T cells. *A*, Identification of the optimal epitope of HLA-B*18-restricted $CD8^+$ T cells. Peripheral blood mononuclear cells (PBMCs) from an HLA-B*18-positive individual chronically infected with human immunodeficiency virus type 1 (HIV-1) were stimulated with NY9 peptide and cultured for 2 weeks. Recognition of the bulk $CD8^+$ T cells toward each peptide was measured by the intracellular cytokine staining (ICS) assay. *B*, Induction of NY8-specific $CD8^+$ T cells in HLA-B*18-positive individuals chronically infected with HIV-1. PBMCs from 8 chronically HIV-1-infected HLA-B*18-positive individuals were stimulated with NY9 peptide and cultured for 2 weeks. Recognition of the bulk $CD8^+$ T cells toward NY8 peptide were measured by the ICS assay. *C*, Effects of E138G/A/K substitutions on the recognition of HLA-B*18-restricted $CD8^+$ T cells. Recognition of the bulk $CD8^+$ T cells toward each wild-type or mutant peptide was measured by the ICS assay. Abbreviations: IFN-γ, interferon gamma; NY8, NETPGIRY; NY8-2G, NGTPGIRY; NY8-2A, NATPGIRY; NY8-2 K, NKTPGIRY; NY9, NNETPGIRY.

Table 2. Susceptibility of Recombinant HIV-1 Variants to 4 Nonnucleoside Reverse Transcriptase Inhibitors

	IC_{50} (nM), Fold Resistance[a]			
Amino Acid	EFV	NVP	ETR	RPV
E138 (wild-type)	1.2 ± 0.2 (1)	31 ± 3 (1)	1.1 ± 0.1 (1)	0.16 ± 0.04 (1)
E138G	1.6 ± 0.2 (1.3)	30 ± 10 (0.97)	2.4 ± 0.3 (2.2)	0.82 ± 0.09 (5.1)
E138A	2.1 ± 0.3 (1.8)	30 ± 2 (0.97)	2.6 ± 0.2 (2.4)	1.13 ± 0.20 (7.1)
E138K	2.4 ± 0.4 (2.0)	50 ± 10 (1.6)	2.4 ± 0.1 (2.2)	0.43 ± 0.10 (2.7)

Data are presented as mean ± standard deviation.

Abbreviations: EFV, efavirenz; ETR, etravirine; IC_{50}, viral 50% inhibitory concentration; HIV-1, human immunodeficiency virus type 1; NVP, nevirapine; RPV, rilpivirine.

[a] Fold resistance was calculated by comparing viral IC_{50} with that of monoclonal wild-type HIV-1.

previous reports [15, 16]. In brief, the initial models of wild-type RT with rilpivirine were first constructed by homology modelling. The crystal structures of RT with NNRTI (PDB code: 2ZD1 [17]) was used for template structure. We also constructed the respective mutant RTs with rilpivirine by considering every possible conformer of the respective mutant models. The possible conformers were generated from the wild-type homology models using PyMOL software (http://www.pymol.org). Among the conformers, we selected those with the lowest energy as each mutant model.

RESULTS

First, we analyzed the frequency of amino acid variations at the 138th position of HIV-1 RT in 1107 ART-naive individuals. As expected, E138 was found in the majority (1099 cases [99%]) of the analyzed patients. However, 8 cases showed amino acid substitutions, including 3 cases of substitution with glycine (E138G), 3 cases with alanine (E138A), and 2 cases with lysine (E138 K). The frequency of E138G/A/K substitutions was 21% and 0.37% in 19 and 1088 individuals with and without HLA-B*18, respectively (Table 1). There was a significant difference in the frequency of the substitutions (odds ratio, 72.3; $P = 4.9 \times 10^{-25}$), suggesting that E138G/A/K could be selected by HLA-B*18-restricted CTLs.

Next, we delineated the impact of E138G/A/K on the response of HLA-B*18-restricted CTLs. The putative HLA-B*18-restricted CTL epitopes containing the 138th position of HIV-1 RT were NETPGIRYQY (NY10; position 137–146), NETPGIRYQ (NQ9; position 137–145), and NNETPGIRY (NY9; position 136–144) [10, 11]. These 3 peptides were used to stimulate PBMCs of 8 ART-treated HLA-B*18-positive patients chronically infected with HIV-1. IFN-γ production activity was detected in PBMCs from 1 of the 8 patients when stimulated with NY9. To determine the optimal epitope, the bulk $CD8^+$ T cells were further analyzed for NY9 and NETPGIRY (NY8; position 137–144). The bulk $CD8^+$ T cells more efficiently recognized NY8 than NY9 at 1-nM, 10-nM, and 100-nM concentrations (Figure 1*A*). These findings indicate that NY8 was the optimal epitope of HLA-B*18-restricted CTLs. Indeed, NY8-specific $CD8^+$ T cells were induced in 3 of the 8 patients (Figure 1*B*). A

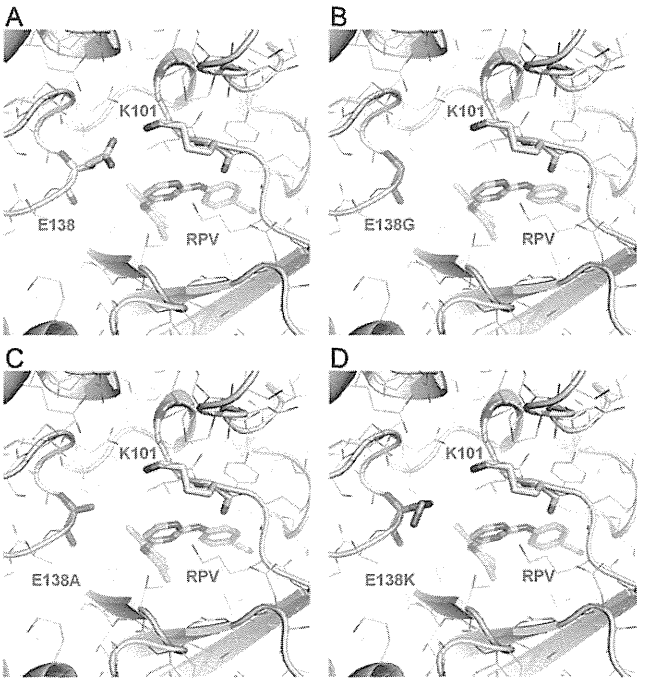

Figure 2. Structural models of human immunodeficiency virus type 1 reverse transcriptase (RT) and rilpivirine. The binding clefts of 4 complexes are shown: $RT_{E138(wild\text{-}type)}$ (*A*), RT_{E138G} (*B*), RT_{E138A} (*C*), and RT_{E138K} (*D*). Sticks indicate the amino acids at positions 101 and 138 of RT, and the atoms of rilpivirine. The mutated residues (E138G, E138A, and E138 K) and rilpivirine atoms are represented by orange and greenish-blue sticks, respectively. Abbreviation: RPV, rilpivirine.

previous study showed that HLA-B*18-binding peptides have 2 anchor residues, E at position 2 and Y/F at the C-terminus [18]. NY8 also had these 2 anchor residues, supporting that this peptide is a HLA-B*18-restricted CTL epitope. To analyze the effect of E138G/A/K on the CTL response, 3 mutant peptides, NGTPGIRY (NY8-2G), NATPGIRY (NY8-2A), and NKTPGIRY (NY8-2 K), were synthesized, and the recognition of the bulk CTLs for these mutant peptides was compared with that for NY8. The bulk CTLs failed to recognize these peptides at 0.1-nM, 1-nM, 10-nM, and 100-nM concentrations, although it effectively recognized NY8 (Figure 1*C*). These substitutions at the 138th position may affect peptide binding to the HLA-B*18 molecule because the second position of HLA-B*18-binding peptides is an anchor for HLA-B*18 [18]. These findings indicate that each of the E138G/A/K affected CTL recognition and allow escape from the HLA-B*18-restricted CTLs.

Finally, we analyzed the effect of E138G/A/K on viral susceptibility to NNRTIs by constructing recombinant HIV-1 variants. Each HIV-1 variant harboring one of E138G/A/K showed comparable replication fitness with wild-type HIV-1. Although the substitutions of E138G/A/K did not confer >2-fold resistance to efavirenz and nevirapine, they conferred mild resistance (2.2- to 2.4-fold) to etravirine. With regard to rilpivirine, E138 K, which was commonly observed in patients with virological failure under rilpivirine-based ART [1, 2], conferred mild resistance, whereas E138G and E138A conferred >5-fold resistance (Table 2). These findings indicate that in addition to E138 K, E138G and E138A can also reduce the clinical response to rilpivirine. The structural modeling suggests that substitution of E138 changes interactions around the rilpivirine-binding cleft (Figure 2). The side chain of E138 in the wild-type RT forms a salt bridge with the lysine at the 101th position (K101) at the edge of the cleft and establishes direct interactions with the pyrimidine moiety of rilpivirine, as seen in the crystal structure of RT with rilpivirine [17]. Meanwhile, mutant RTs with E138G/A/K substitutions could not create such a salt bridge, resulting in changes in the morphology of the binding cleft. In particular, RTs with E138G or E138A can reduce interactions with rilpivirine by creating large gaps between rilpivirine and the substituted 138th residues with small side chains, which seems to cause significant resistance to rilpivirine.

DISCUSSION

The major findings of the present study were as follows: (1) E138G/A/K substitutions were escape mutations of HLA-B*18-restricted CTLs and they were observed more frequently in HLA-B*18-positive patients than HLA-B*18-negative patients; and (2) we confirmed that these substitutions conferred significant resistance to rilpivirine, demonstrating that drug resistance–associated mutations can be selected naturally by CTL when its epitope is located in the viral protein of antiretroviral targets.

Studies of cellular immunology in HIV-1 have focused mainly on Gag [19, 20]. However, considering that many of the recently identified CTL epitopes are located in Pol [13, 14, 21], analysis of the interaction between CTL and drug susceptibility is warranted. Some escape mutations can persist after viral transmission to other hosts even if the new hosts do not have the corresponding HLAs [22]. Therefore, HIV-1 can adapt to HLA at a population level [23]. In fact, we identified E138G/A/K in ART-naive HLA-B*18-negative patients, although the frequency of such variations was extremely low. However, the same analysis performed in areas with higher prevalence of HLA-B*18, such as Eastern Europe [24], would probably detect higher frequency of E138G/A/K.

HIV drug resistance testing is recommended not only after treatment failure but also before the introduction of the initial treatment, considering the risk that the patient may have acquired drug-resistant viruses from those with treatment failure [3, 25]. The present study may add another reason for drug resistance testing of ART-naive patients: drug resistance–associated mutations may have evolved in the patients selected by their own immunity even if the original transmitted viruses were drug sensitive. At the very least, drug resistance testing should be performed before the introduction of rilpivirine-based ART in HLA-B*18-positive patients.

Notes

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Preemptive Therapy Prevents Cytomegalovirus End-Organ Disease in Treatment-Naïve Patients with Advanced HIV-1 Infection in the HAART Era

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Abstract

Background: The efficacy of preemptive therapy against cytomegalovirus (CMV) infection remains unknown in treatment-naïve patients with advanced HIV-1 infection in the HAART era.

Methods: The subjects of this single-center observation study were126 treatment-naïve HIV-1 infected patients with positive CMV viremia between January 1, 2000 and December 31, 2006. Inclusion criteria were age more than 17 years, CD4 count less than 100/μl, plasma CMV DNA positive, never having received antiretroviral therapy (ART) and no CMV end-organ disease (EOD) at first visit. The incidence of CMV-EOD was compared in patients with and without preemptive therapy against CMV-EOD. The effects of the CMV preemptive therapy were estimated in uni- and multivariate Cox hazards models.

Results: CMV-EOD was diagnosed in 30 of the 96 patients of the non-preemptive therapy group (31%, 230.3 per 1000 person-years), compared with 3 of the 30 patients of the preemptive therapy group (10%, 60.9 per 1000 person-years). Univariate (HR = 0.286; 95%CI, 0.087–0.939; p = 0.039) and multivariate (adjusted HR = 0.170; 95%CI, 0.049–0.602; p = 0.005) analyses confirmed that CMV-EOD is significantly prevented by CMV preemptive therapy. Multivariate analysis showed that plasma CMV DNA level correlated significantly with CMV-EOD (per log10/ml, adjusted HR = 1.941; 95%CI, 1.266–2.975; p = 0.002). Among the 30 patients on preemptive therapy, 7 (23.3%) developed grade 3–4 leukopenia. The mortality rate was not significantly different between the two groups (p = 0.193, Log-rank test).

Conclusions: The results indicate that preemptive therapy lowers the incidence of CMV-EOD by almost 25%. Preemptive therapy for treatment-naïve patients with CMV viremia is effective, although monitoring of potential treatment-related side effects is required.

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Introduction

Although the incidence of new cases of cytomegalovirus (CMV) end-organ disease (EOD) has decreased by 75%–80% with the advent of antiretroviral therapy (ART) and is currently estimated to be <6 cases per 100 person-years [1], CMV-EOD is still one of the major debilitating diseases among patients with advanced HIV infection.

CMV preemptive therapy is commonly used for patients scheduled for hematopoietic cell transplantation and solid organ transplantation, with clinical evidence of efficacy[2–6], however, it is not generally recommended in HIV patients [7] because of concerns regarding cost-effectiveness, risk of developing CMV resistance, side effect and the lack of a proven survival benefit [8]. A prospective trial in cooperation with Roche company to evaluate the efficacy of preemptive therapy in the pre-HAART (highly active ART) era showed significant preventive effect of oral ganciclovir (GCV) [9]. However; other studies conducted in both pre-HAART and HAART eras showed no significant effect [10,11]. However, the above studies included patients who had previously received ART. Therefore, the efficacy of preemptive therapy against CMV infection remains unknown in treatment-naïve patients with advanced HIV-1 infection in the HAART era.

We retrospectively compared the incidence of CMV-EOD in a cohort of ART-naïve adult patients with advanced HIV infection (low CD4 count and plasma CMV-DNA-positive). One group of these patients had received CMV preemptive therapy, while the other had not received such therapy.

Methods

Ethics Statement

The study was approved by the Human Research Ethics Committee of National Center for Global Health and Medicine,

Tokyo. All patients included in this study provided a written informed consent for their clinical and laboratory data to be used and published for research purposes. This study has been conducted according to the principles expressed in the Declaration of Helsinki.

Study design

We performed a retrospective, single-center cohort study to elucidate the effectiveness of preemptive CMV treatment in HIV-infected patients with positive CMV viral load in the prevention of CMV-EOD. The study was conducted at the National Center for Global Health and Medicine, Tokyo, one of the largest clinics for patients with HIV infection in Japan, with more than 2,700 registered patients as of December 2006. The study population comprised treatment-naïve HIV infected patients aged more than 17 years, with CD4 count less than 100/μl and positive plasma CMV DNA viral load, who presented for the first time at our hospital between January 1, 2000 and December 31, 2006. Those with CMV-EOD at presentation and those with <3 months of follow-up were excluded. The follow-up period was 2 years from the initial visit.

Definition of CMV-EOD and CMV preemptive therapy

CMV-EOD was diagnosed according to standardized ACTG criteria (see Table S1) [11]. CMV retinitis was routinely screened for by dilated indirect ophthalmoscopy at both the first visit to the hospital and a few months after the commencement of ART. Other evaluations, such as endoscopy and bronchoscopy, were carried out in response to the symptoms and clinical condition. The diagnosis of CMV-EOD was established by at least two experts from our hospital.

CMV preemptive therapy was prescribed based on the clinician's assessment. CMV preemptive therapy was provided at our institution for patients with plasma CMV DNA of >5000 copies/ml. For patients with plasma CMV DNA of >3000 but less than 5000 copies/ml, the decision to initiate preemptive therapy was left to the attending physician, taking into consideration the overall clinical condition, such as subsequent rise in plasma CMV DNA and/or use of immunosuppressants, such as steroids and chemotherapeutic agents. Ganciclovir (GCV) and valganciclovir (VGCV) were the most commonly used agents, followed by foscarnet (FOS). The choice of induction (intravenous GCV 5 mg/kg every 12 hours, oral VGCV900 mg twice a day or intravenous FOS 90 mg/kg every 12 hours) or maintenance dose (intravenous GCV 5 mg/kg every 24 hours, oral VGCV 900 mg a day or intravenous FOS 90 mg/kg every 24 hours) was based on the clinical condition, such as the level of plasma CMV DNA or state of immunosuppression. The duration of therapy varied across individuals. CMV preemptive therapy was defined as at least a 7-day treatment with agents effective against CMV. The normal course of CMV preemptive therapy was 2 weeks of GCV induction dose followed by VGCV or GCV maintenance dose until plasma CMV DNA became negative. Patients were retreated based on clinicians' decision under some conditions with high risks for CMV-EOD as described above, if plasma CMV DNA became positive again after preemptive therapy.

Measurements

Plasma CMV DNA was measured using real-time PCR with a lower limit of detection of 200 copies/mL(CMV geniQ, Bio Medical Laboratory, Inc., Tokyo, Japan). Plasma CMV DNA was measured routinely at the first visit in patients with CD4 count of <100/μl, and re-examined every week or monthly, according to the level of plasma CMV DNA viral load or immune status and at the discretion of the attending physician.

In this study, the primary exposure variable was CMV preemptive therapy over no CMV preemptive therapy. The potential risk factors for CMV-EOD were determined based on previous studies [12–18], and included basic demographics and laboratory data, including age, sex, CD4 cell count, HIV viral load, plasma CMV DNA, and presence or absence of other medical conditions (concurrent use of steroids, concurrent chemotherapy and concurrent AIDS-defining diseases). For each patient, data on or closest to the day of the first visit to our hospital were retrieved for analysis.

Statistical analysis

Categorical and continuous baseline demographics and laboratory data were analyzed using Pearson's chi-square test and Student's t-test, respectively. The time from the first visit to our hospital to the development of CMV-EOD was analyzed by the Kaplan Meier method for patients on CMV preemptive therapy and no CMV preemptive therapy, and the log-rank test was used to determine the statistical significance. Censored cases represented those who died, dropped out, or were referred to other facilities before the end of follow-up period. The Cox proportional hazards regression analysis was used to estimate the impact of CMV preemptive therapy on the incidence of CMV-EOD. The impact of basic demographics, baseline laboratory data, and other medical conditions was also estimated with univariate Cox proportional hazards regression.

To estimate the unbiased prognostic impact of CMV preemptive therapy, we used three models based on multivariate Cox proportional hazards regression analysis. Model 1 was the aforementioned univariate analysis for CMV preemptive therapy. Model 2 included age and sex, plus Model 1, in order to adjust for basic characteristics. In Model 3, we added variables with significant relation to CMV-EOD by univariate analysis or assumed as risk factor(s) for CMV-EOD in the literature[12–20] (e.g., CD4 count per 1/μl decrement, HIV viral load per log10/ml, CMVDNA viral load per log10/ml, concurrent steroid use, concurrent chemotherapy and concurrent AIDS defining disease). Statistical significance was set at two-sided p values <0.05. We used hazard ratios (HRs) and 95% confidence intervals (95%CIs) to estimate the impact of each variable on CMV-EOD. All statistical analyses were performed with The Statistical Package for Social Sciences ver. 17.0 (SPSS, Chicago, IL).

Results

Of the 199 HIV-infected patients with CD4 count <100/μl and positive plasma CMV DNA viral load referred to our hospital between January 1, 2000 and December 31, 2006, 126 patients were recruited in the study. Of these, 96 patients received CMV preemptive therapy while 30 did not (Figure 1). Table 1 lists the demographics, laboratory data, and medical conditions of the study population at baseline. The majority of the study population were males, East Asians, and relatively young (median: 42 years). There were no differences in baseline CD4 count (p = 0.595) and HIV viral load (p = 0.628) between the two groups. Patients of the CMV preemptive therapy group had higher plasma CMV DNA viral load (p<0.001), more likely to have developed AIDS defining diseases (p = 0.042), and tended to have been treated concurrently with steroids (p = 0.009), compared with the non-CMV preemptive group. There were no significant differences in the use of chemotherapy (p = 1.000) and in time to initiation of ART since study entry (p = 0.393, Table 1) between the two groups.

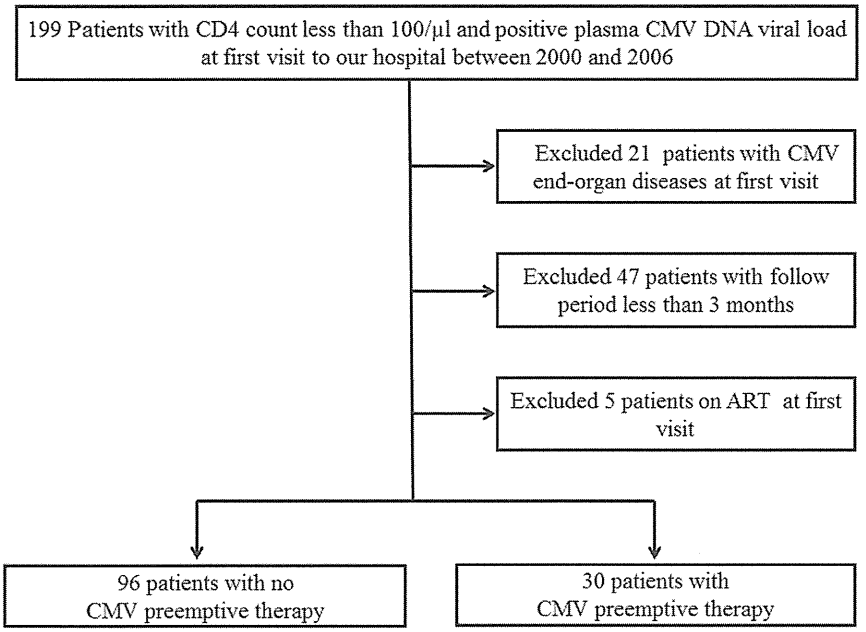

Figure 1. Flow chart of inclusion and exclusion criteria. Of the 199 subjects, 73 were excluded and the remaining 126 were included in the study. The latter group was divided into the preemptive therapy group (n = 30) and the non-therapy group (n = 96).
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During the follow-up period, CMV-EOD occurred in 3 (10.0%) patients of the preemptive therapy group and 30 (31.3%) of the non-preemptive therapy group, with an estimated incidence of 60.9 and 230.3 per 1000 person-years, respectively. Figure 2 depicts the time from the first visit to our hospital to the development of CMV-EOD by Kaplan Meier method in the two groups. The incidence of new cases of CMV-EOD was significantly higher in the non-preemptive therapy group, compared with the preemptive therapy group ($p = 0.027$, Log-rank test). The median time from the first visit to the diagnosis of CMV-EOD was 67 days (range, 25–67) for the preemptive therapy group, and 54 days (range, 14–326 days) for the non-preemptive therapy group.

Univariate analysis showed a significant relationship between CMV preemptive therapy and low incidence of CMV-EOD (HR = 0.286; 95%CI, 0.087–0.939; $p = 0.039$) (Table 2). On the other hand, high CMV viral load and HIV viral load tended to be associated with CMV-EOD, while old age, low baseline CD4

Table 1. Baseline demographics and laboratory data of patients who did and did not receive CMV preemptive therapy.

	Non-preemptive therapy (n = 96)	Preemptive therapy (n = 30)	P value
Sex (male), n (%)	88 (91.7)	29 (96.7)	0.685
Median (range) age	41 (24–76)	44 (25–66)	0.729
Ethnicity, n (%)			
East Asians	86 (89.5)	29 (96.7)	
Southeast Asian	5 (5.2)	0 (0.0)	
Black	3 (3.1)	0 (0.0)	
White	2 (2.1)	1 (3.3)	
Median (range) CD4 count (/μl)	28.0 (0–97)	35.5 (3–87)	0.595
Median (range) HIV RNA viral load (log10/ml)	5.3 (3–6)	5.35 (4–7)	0.628
Median (range) CMVDNA viral load (log10/ml)	3.0 (2–5)	4.3 (2–5)	<0.001
Concurrent AIDS, n (%)	78 (81.3)	29 (96.7)	0.042
Steroid use, n (%)	38 (39.6)	20 (66.7)	0.009
Chemotherapy, n (%)	9 (9.4)	2 (6.7)	1.000
Median (range) time (days) to ART*	66 (2–399)	59 (13–158)	0.393
Median (range) follow-up (days)	730 (14–730)	730 (25–730)	0.064

*11 missing values.
Categorical and continuous variables were analyzed using Pearson's chi-square test and Student's t-test, respectively.
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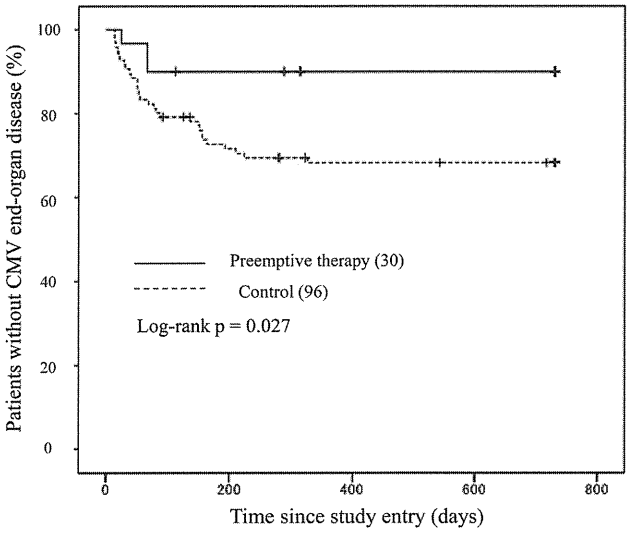

Figure 2. Kaplan-Meier curve showing the time to development of cytomegalovirus (CMV)- end-organ disease (EOD) in the preemptive and non-preemptive therapy groups. Compared to patients on CMV preemptive therapy, those who did not receive preemptive therapy were more likely to develop CMV-EOD (p = 0.027, Log-rank test).
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count, use of steroids, chemotherapy, and concurrent AIDS defining diseases were not associated with CMV-EOD. Multivariate analysis identified CMV preemptive therapy as a significant preventive factor against CMV-EOD after adjustment for age and sex (Model 2; adjusted HR = 0.289; 95%CI, 0.088–0.949; p = 0.041, Table 3), and after adjustment for other risk factors (Model 3; adjusted HR = 0.172; 95%CI, 0.049–0.602; p = 0.005, Table 3). In addition, multivariate analysis showed that high CMV viral load correlated significantly with CMV-EOD (Model 3; adjusted HR = 1.941; 95%CI, 1.266–2.975; p = 0.002, Table 3).

Of the 33 patients with CMV-EOD, 22 (66.7%) developed CMV retinitis, 4 (12.1%) developed esophagitis, 3 (9.1%) developed gastroduodenitis, 6 (18.2%) developed colitis and 1 (3.0%) developed pneumonitis. All 3 patients with CMV-EOD of the preemptive therapy group developed retinitis (Table 4).

Table 2. Results of univariate analysis to estimate the risk of various factors in inducing CMV end-organ disease.

	Hazard ratio	95% CI	P value
CMV preemptive therapy	0.286	0.087–0.939	0.039
Female	1.284	0.392–4.209	0.680
Age per 1 year	0.982	0.951–1.013	0.240
CD4 count per 1/μl decrement	1.001	0.989–1.013	0.867
HIV viral load per log10/ml	1.875	0.905–3.884	0.091
CMV viral load per log10/ml	1.450	0.984–2.136	0.060
Use of steroid	0.716	0.356–1.439	0.348
Chemotherapy	1.390	0.488–3.955	0.537
Concurrent AIDS	0.703	0.290–1.704	0.436

CI: confidence interval
The Cox proportional hazards regression analysis was used.
doi:10.1371/journal.pone.0065348.t002

Of 30 patients who received preemptive therapy, 20 (66.7%) received an induction dose of GCV, and 7 patients (23.3%) received maintenance dose. The remaining agents used for preemptive therapy were an induction dose of VGCV, a maintenance dose of FOS and an induction dose of cidofovir. The duration of the preemptive therapy varied between 7 days and 2 months. The following side effects were noted in patients on CMV preemptive therapy: grade 3/4 leukopenia (n = 7, 23.3%) and grade 2 hypercreatininemia (n = 1, 3.3%). Both side effects developed during the use of GCV. Five patients (5.2%) of the non-preemptive therapy group and 4 patients (13.3%) of the preemptive therapy group died during the study period. Of the former group, 3 deaths were due to opportunistic infections (cryptococcus meningitis, non-tuberculous mycobacterial infection and *Pneumocystis jiroveci* pneumonia), 1 due to bacterial infection (sepsis), and 1 due to suicide. Of the latter group, 2 deaths were due to opportunistic infections (malignant lymphoma and *P. jiroveci* pneumonia) and 2 due to bacterial infection (bacterial pneumonias). Deaths and bacterial infections related to preemptive therapy were not observed in our study. The mortality rate was not significantly different between the two groups (p = 0.193, Log-rank test, Figure 3).

Discussion

The results of this observational cohort of treatment-naïve HIV-infected patients with positive plasma CMV DNA showed a significantly lower incidence of CMV-EOD by one-fourths in the CMV preemptive therapy group than in the non-preemptive therapy group, over the 2-year observation period. This finding was significant, despite higher risk for CMV-EOD in the preemptive therapy group, such as higher plasma CMV DNA, higher prevalence of concurrent AIDS defining diseases and more concurrent steroid use, compared with the other group. Univariate and multivariate analyses identified anti-CMV preemptive therapy as a significant preventive factor against CMV-EOD.

Our study is the first to illustrate the significance of anti-CMV preemptive therapy in treatment- naïve HIV-infected patients with CMV viremia and CD4 count less than 100/μl in the HAART era. The hazard ratio of development of CMV-EOD decreased by 82.8% following preemptive therapy, compared with no preemptive therapy, even after adjustment for plasma CMV DNA viral load and other factors. The current guidelines do not generally recommend anti-CMV preemptive therapy although this is based on sparse evidence, such as cost effectiveness, CMV resistance, and drug side effects [7]. However, our study suggests that preemptive therapy is a feasible option, if the effective target of preemptive therapy could be selected. Furthermore, the study confirmed that plasma CMV DNA, a known risk factor for CMV-EOD [12–18], was a significant independent risk factor.

A few prospective clinical trials investigated the efficacy of preemptive therapy in both the pre-HAART era and HAART era. In these studies, oral GCV at 1000 mg thrice daily was used in the pre-HAART era regimen [9,10] while VGCV at 900 mg twice daily was the regimen used in the HAART era [11]. The patients investigated in the above three studies were HIV-treatment-experienced patients. One study in the pre-HAART era reported the efficacy of preemptive therapy in patients with CD4 count<50 μl [9], while the other studies showed no significant preventive effect [10,11]. In the ACTG A5030 study, the prospective clinical trial in the HAART era, which evaluated the efficacy of oral VGCV 900 mg twice a day for 3 weeks among HIV-infected patients with CD4 count <100 cells/mm^3, plasma HIV RNA >400 copies/mL, plasma CMV viremia and on stable

Table 3. Results of multivariate analysis to estimate the preventive effect of CMV preemptive therapy against CMV end-organ disease.

	Model 1 Crude		Model 2 Adjusted		Model 3 Adjusted	
	HR	95% CI	HR	95%CI	HR	95%CI
CMV preemptive therapy*	0.286	0.087–0.939	0.289	0.088–0.949	0.172	0.049–0.602
Age			0.982	0.952–1.014	0.990	0.958–1.022
Female			1.033	0.310–3.441	0.988	0.267–3.653
CD4 count per 1/μl decrement					0.995	0.983–1.008
HIV viral load per log10/ml					2.217	0.912–5.393
CMV viral load per log10/ml*					1.941	1.266–2.975
Use of steroid					0.664	0.288–1.534
Chemotherapy					1.668	0.540–5.151
Concurrent AIDS					0.930	0.337–2.569

*P<0.05 in Model 3
HR: hazard ratio, CI: confidence interval
The Cox proportional hazards regression analysis was used.
Variables with significant difference by univariate analysis or assumed as risk factors for CMV-EOD in the literature were included in model 3.
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or no HAART, the authors reported a low incidence of CMV-EOD among subjects both with and without preemptive therapy [11]. The authors attributed the low incidence to improvement of immune function induced by potent ART. Actually, in that study [11], the number of patients who had received ART at study entry was about 80% of the total. In contrast, the subjects of our study were all treatment-naïve patients and possibly at higher risk for CMV-EOD compared to those enrolled in the ACTG A5030. Thus, the use of CMV preemptive therapy reported in our study under the clinical scenario of poor immune status without ART at study entry resulted in better outcome than in previous studies. In our study, there was no significant difference in the timing of ART between the two treatment groups. Although our study did not include the time to the initiation of ART as a variable in uni- and multivariate analysis because the values for 11 cases were missing, multivariate analysis with the time to the initiation of ART together with other variables similarly identified preemptive therapy as a significant preventive factor (adjusted HR = 0.235; 95%CI, 0.064–0.868; p = 0.030).

The survival benefits of CMV preemptive therapy were controversial in previous prospective clinical trials. One study suggested the survival benefits of 3 g/day oral GCV preemptive therapy [9], while other studies showed no evidence of the survival benefit [10]. On the other hand, two prospective cohort studies in the HAART era showed the relation between CMV viremia and high mortality [21] and suggested the benefit of CMV therapy [22], whereas our results showed no significant difference in mortality rate between the two groups. The reason for this discrepancy could be attributed to low mortality rate, small sample size and the disproportionally high risk of the therapy group in our study. The mortality rate (5.0 deaths per 100 person-years) in our study was similar to that in a study conducted in the HAART era (5.7 deaths per 100 person-years)[19] and was considerably lower than in studies from the pre-HAART era. Since the mortality rate has markedly decreased in advanced HIV infected patients following the introduction of potent ART in the HAART era [23,24], not only the survival benefit but also quality of life, such as improvement of eye function, should be emphasized in the future.

The side effects of preemptive therapy have also been of concern [25]. Our findings showed the development of grade 3 to 4 leukocytopenia in 23.3% of the patients who received intravenous GCV, and was the major side effect of preemptive therapy. Some patients who developed leukocytopenia required treatment with granulocyte colony-stimulating factor (G-CSF) and showed complete recovery. Thus; careful follow-up of patients on preemptive therapy is necessary. For these reasons, preemptive

Table 4. Details of CMV end-organ disease.

CMV-EOD	n (%)	Time to development (days)	Non-preemptive therapy group	Preemptive therapy group
Retinitis	22* (61.1%)	72 (14–326)	19* (57.6%)	3 (100%)
Esophagitis	4* (11.1%)	116.5(69–164)	4* (12.1%)	0
Gastroenteritis	3* (8.3%)	19 (14–40)	3* (9.1%)	0
Colitis	6* (16.7%)	40.5 (15–55)	6* (18.2%)	0
Pneumonitis	1 (2.8%)	31 (31–31)	1 (3.0%)	0
Total	36* (100%)	55 (14–326)	33* (100%)	3 (100%)

*Three patients of the non-preemptive therapy group had multiple CMV-EOD; one with retinitis plus esophagitis, one with retinitis plus gastroenteritis and the other with retinitis plus colitis.
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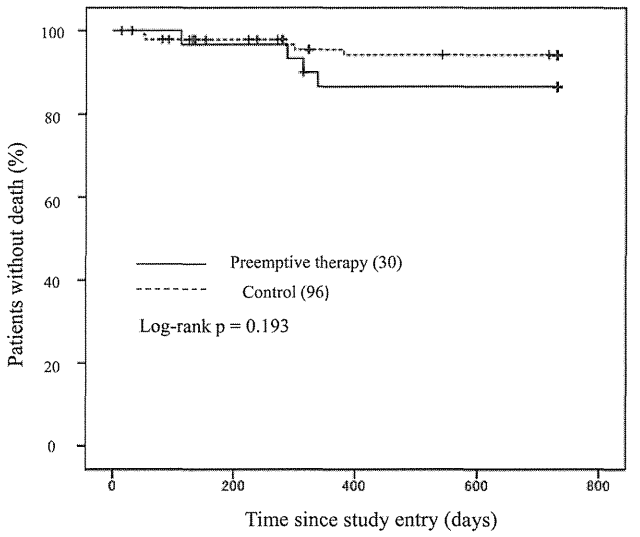

Figure 3. Kaplan-Meier curve showing the time to death in the preemptive and non-preemptive therapy groups. There was no significant difference in the survival rate between the two groups (p = 0.193, Log-rank test).
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therapy might place patients at greater risk in resource-limited setting, where close monitoring is difficult and the risk of bacterial infection is high. It is noteworthy, however, that death and bacterial infection related to preemptive therapy were not observed in our study.

The present study has several limitations. Due to its retrospective nature, it was not possible to control the baseline characteristics of the enrolled patients. However, patients with potential risk for CMV-EOD, such as those with high plasma CMV DNA, high concurrent AIDS and high steroid use, were more likely prescribed the preemptive therapy. It is noteworthy that the incidence of CMV-EOD was significantly lower in the preemptive therapy group despite this adverse environment.

Second, the criteria for treatment, choice of drugs and duration of CMV preemptive therapy were not rigidly controlled in the present study. Thus, it was difficult to determine which anti-CMV agent with what dosage is optimal for preemptive therapy. In the present study, about 90% of patients received induction dose or maintenance dose of GCV since the majority of patients of the preemptive therapy group were in-patients. Further prospective study is required to optimize effective preemptive therapy, including oral VGCV.

Third, CMV-EOD, especially enteritis, could have been overlooked at study entry since routine endoscopic screening was not performed, compared with screening for retinitis at the first visit. However, patients with abdominal pain were subjected to stool examination for occult blood, since the definition of CMV enteritis includes abdominal pain, and those with positive tests were subsequently considered for endoscopy. Thus, the possibility of latent CMV enteritis at study entry does not seem to have affected the results of the present study.

In conclusion, the present study demonstrated a lower incidence of CMV-EOD following CMV preemptive therapy by one-fourth, compared with no preemptive therapy, in treatment-naïve patients with CMV viremia. High plasma CMV DNA was identified as an independent risk for CMV-EOD. Further studies are warranted to elucidate the efficacy, safety and cost-effectiveness of anti-CMV preemptive therapy in HIV infected patients at high risk for EOD.

Supporting Information

Table S1 Definitions of CMV end-organ diseases used in this study.
(DOCX)

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Author Contributions

Conceived and designed the experiments: DM K. Tsukada K. Teruya. Performed the experiments: DM TN K. Teruya. Analyzed the data: DM HG YK SO. Contributed reagents/materials/analysis tools: YK K. Tsukada. Wrote the paper: DM TN HG SO.

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