TABLE 1. PATIENT CHARACTERISTICS

	Diagnosis (month)	*Sex*	*Age*	*VL*	*CD4*	*CD8*	*Treatment period (month)*
19 LVL							
S70	*9*	*M*	*47*	*53*	*481*	*746*	
T16	*60*	*F*	*41*	*240*	*492*	*804*	
O12	*61*	*M*	*32*	*450*	*559*	*1,187*	
S33	*125*	*M*	*52*	*470*	*400*	*888*	
K2	*60*	*M*	*30*	*510*	*316*	*871*	
M3	*160*	*F*	*34*	*730*	*444*	*859*	
S81	*6*	*M*	*32*	*1,700*	*381*	*1,475*	
Y1	*131*	*M*	*36*	*1,700*	*358*	*753*	
F4	*101*	*M*	*32*	*2,000*	*404*	*699*	
E6	*13*	*M*	*29*	*2,100*	*348*	*649*	
T24	30	M	46	400	455	469	
O16	23	M	36	1,100	362	812	
F9	65	M	35	1,100	517	1,066	
K11	33	M	30	1,200	521	1,137	
F1	41	M	35	1,600	424	1,151	
H25	6	M	43	1,700	749	821	
K16	61	F	23	2,000	560	1,049	
A10	32	M	25	3,400	586	1,603	
T26	28	M	36	3,600	449	864	
median	41		35	1,200	449	868	
16 HVL							
S60	*22*	*M*	*36*	*35,000*	*321*	*486*	
H24	*23*	*M*	*35*	*42,000*	*462*	*907*	
F13	*22*	*M*	*34*	*51,000*	*520*	*831*	
O29	*9*	*M*	*21*	*56,000*	*314*	*880*	
Y24	*14*	*M*	*25*	*58,000*	*381*	*2,023*	
K54	*10*	*M*	*24*	*82,000*	*386*	*1,056*	
K43	*11*	*M*	*29*	*110,000*	*361*	*507*	
S78	*5*	*M*	*48*	*260,000*	*492*	*1,441*	
K46	*15*	*M*	*47*	*280,000*	*454*	*1,579*	
S55	*26*	*M*	*56*	*500,000*	*427*	*510*	
T37	3	M	30	25,000	254	626	
K33	24	M	39	27,000	314	832	
M11	58	M	38	33,000	228	908	
O17	24	M	33	66,000	494	632	
T35	4	M	20	78,000	434	1,133	
S5	49	M	62	85,000	516	1,029	
median	18.5		35	62,000	407	894	
15 Tx							
U5	*22*	*M*	*42*	*30*	*308*	*581*	*22*
S19	*82*	*M*	*35*	*30*	*382*	*533*	*22*
T18	*70*	*M*	*40*	*40*	*508*	*747*	*66*
T8	*39*	*M*	*42*	*50*	*423*	*491*	*51*
K4	*73*	*M*	*31*	*50*	*480*	*718*	*71*
Y17	*33*	*M*	*36*	*50*	*365*	*508*	*31*
I9	*90*	*M*	*50*	*50*	*406*	*643*	*80*
I5	*59*	*M*	*44*	*50*	*466*	*668*	*56*
N11	*119*	*M*	*39*	*67*	*440*	*886*	*117*
N17	*60*	*M*	*29*	*67*	*382*	*571*	*46*
K24	150	M	54	40	610	805	149
N5	72	M	37	45	633	706	41
O9	61	M	41	50	335	629	72
Y5	81	M	36	110	753	605	79
S15	113	M	38	130	814	1,048	111
median	72		39	50	440	643	66

Italics indicates the patients used for phenotype and activation/exhaustion status of T cells.
VL, viral load; LVL, low viral load; HVL, high viral load; Tx, treatment experienced.

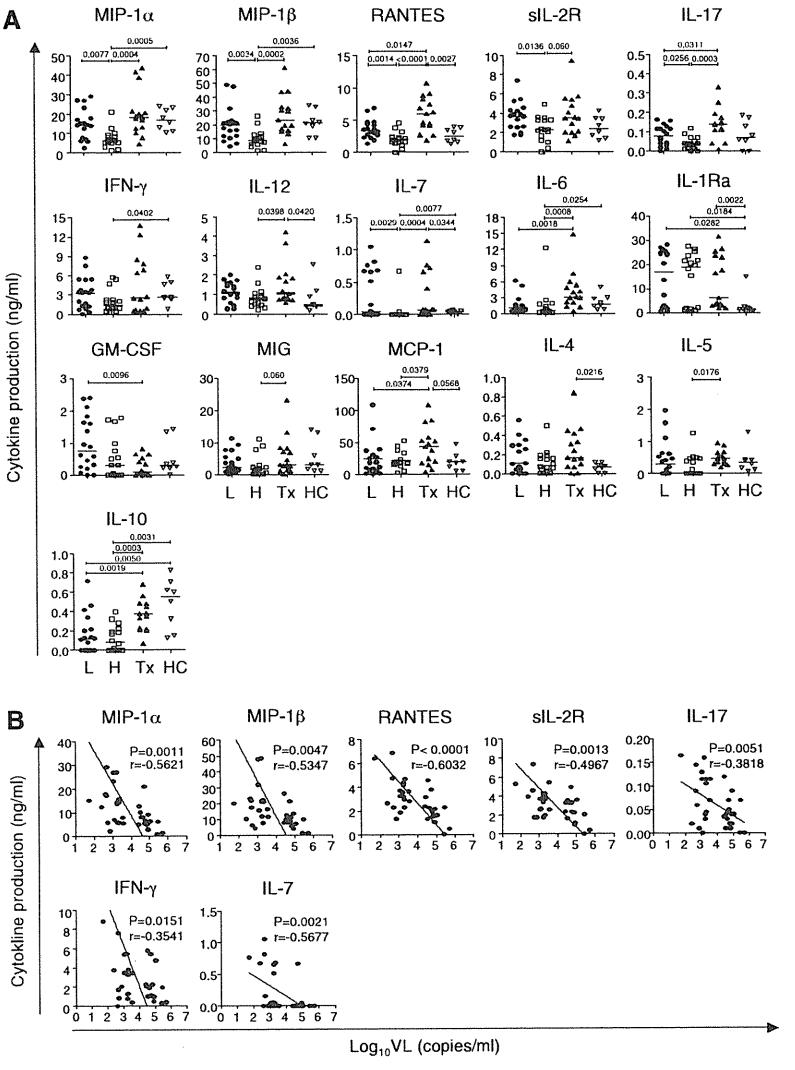


FIG. 1. Multiple cytokine production of phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMCs) in chronic HIV-1-infected subjects and healthy individuals. **(A)** Comparison of cytokine production from PHA-stimulated PBMCs. L, LVL subjects (●), H, HVL subjects (□), Tx, HIV-1-infected subjects with prolonged antiretroviral therapy (ART) (▲), HC, healthy control (□). The horizontal bars indicate the median value. Differences between groups were tested for statistical significance by the Mann–Whitney *U* test. **(B)** Correlation between cytokine production and viral load. Correlation analysis was performed with Spearman's rank correlation to determine correlations between variables.

PHA requires accessory cells such as monocytes and macrophages,[23,24] these data indicate that $CD4^+$ and $CD8^+$ T cells are the sources of MIP-1α, MIP-1β, RANTES, IFN-γ, and sIL-2R production, and that only $CD4^+$ T cells are the source of IL-17.

$CD4^+$ T cells were classified into subsets based on cytokine secretion.[14,16] We found VL-associated reductions in levels of IFN-γ and IL-17 levels, which are typical cytokines secreted by Th1 and Th17 cells, respectively. In contrast, as shown in Fig. 1A, the LVL and HVL groups had comparable levels of IL-4, IL-5, and IL-13, which are associated with a Th2-type response, and IL-10, which is produced by regulatory T cells (Treg) (Fig. 1A). These results suggest that $CD4^+$ T cell dysfunction in HVL may occur in a type-specific manner, especially in Th1 and Th17 cells.

To determine which types of T cells could secrete MIP-1α, MIP-1β, and RANTES under our experimental conditions, we examined the expression pattern of MIP-1α/MIP-1β/RANTES, IFN-γ, and IL-17 by intracellular cytokine staining (ICS) after nonspecific T cell stimulation. Production of MIP-1α/MIP-1β/RANTES occurred in IFN-γ-expressing $CD4^+$ T cells, particularly in the subset of cells that expressed high levels of IFN-γ (Fig. 2B left). IL-17 was also produced in $CD4^+$ T cells, but was secreted by a different $CD4^+$ T cell subset. In $CD8^+$ T cells, most IFN-γ-expressing cells produced MIP-1α/MIP-1β/RANTES, and IL-17 was not produced at all (Fig. 2B right). Thus, our assays showed that MIP-1α, MIP-1β, and RANTES are secreted from Th1-type $CD4^+$ T cells and $CD8^+$ T cells, and that IL-17-secreting cells (Th17 cells) are clearly distinct. These data suggest that cytokine production by T

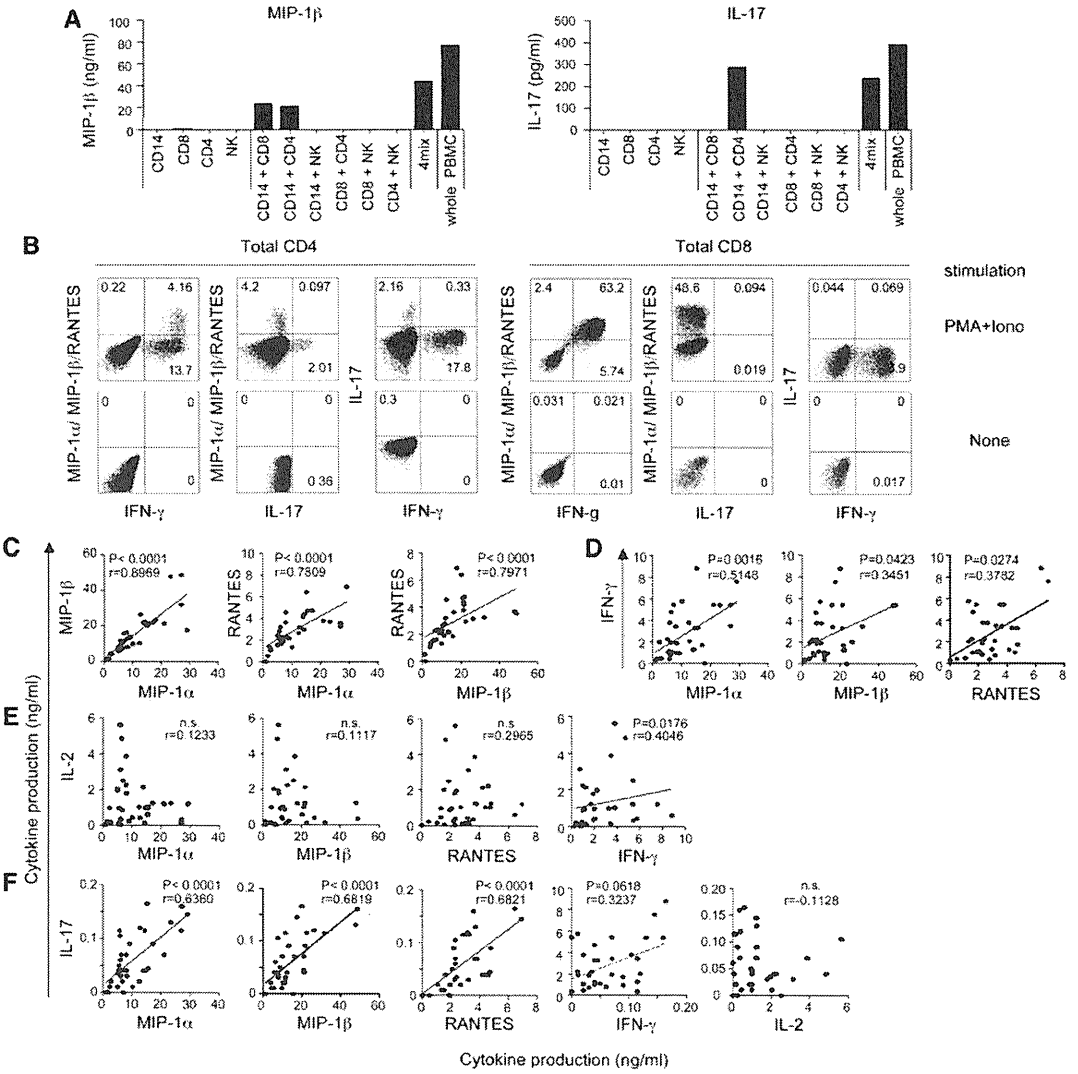


FIG. 2. Identification of cytokine-producing cells and relationships between cytokines. (**A**) Cytokine production in cell fractions from PBMCs. The results of MIP-1β and IL-17 production are shown. Fractionated $CD4^+$, $CD8^+$, $CD14^+$, and NK ($CD56^+CD16^+$) cells from PBMCs in healthy individuals were cultured separately or cocultured for 48 h after PHA stimulation. The experiment was repeated twice with PBMCs from different donors. (**B**) Representative flow cytometric analysis of intracellular cytokine staining for MIP-1α, MIP-1β, RANTES, IFN-γ (Th1 cytokine), and IL-17 after PMA/ionomycin stimulation in PBMCs of healthy individual. (**C–F**) Correlation between each cytokine production in treatment-naive HIV-1-infected subjects. MIP-1α, MIP-1β, and RANTES production (**C**), IFN-γ production and MIP-1α, MIP-1β, or RANTES production (**D**), IL-2 production and MIP-1α, MIP-1β, RANTES, or IFN-γ production (**E**), IL-17 production and MIP-1α, MIP-1β, RANTES, IFN-γ, or IL-2 production (**F**) are shown. Correlation analysis was performed with Spearman's rank correlation to determine correlations between variables.

cells from the HVL group is dysfunctional, specifically in some of the Th1-related cytokines and in IL-17.

We next analyzed the correlation between production of Th1 cytokines (IFN-γ and IL-2), MIP-1α/MIP-1β/RANTES, and IL-17 in treatment-naive HIV-1 subjects. The levels of MIP-1α, MIP-1β, and RANTES showed strong positive correlations to each another (Fig. 2C), and correlations between IFN-γ and each of them were also significant (Fig. 2D). However, IL-2, another typical Th1 cytokine, did not show any significant correlation with MIP-1α, MIP-1β, and RANTES (Fig. 2E). Surprisingly, we found strong correlations between IL-17 production and MIP-1α/MIP-1β/RANTES

levels or IFN-γ levels, despite the fact that these cytokines are produced by different cells (Fig. 2F). These data suggest interrelated production of IFN-γ, MIP-1α, MIP-1β, RANTES, and IL-17 in T cells, but not of IL-2. Moreover, the capacity of T cells to produce these cytokines appears to be affected by HIV-1 VL *in vivo*.

Both central and effector memory CD4⁺ and CD8⁺ T cells are highly activated and exhausted in HVL subjects

The mechanism underlying the reduction in levels of specific cytokines in the HVL group could result either from decreased numbers of the cytokine-producing cells or from decreased productive capacity in those cells. We quantitated $CD4^+$ and $CD8^+$ T cells in HIV-1-infected patients (Table 1) and healthy control subjects (data not shown) by FACS. Although the number of $CD4^+$ T cells was significantly higher and the number of $CD8^+$ T cells significantly lower in HC than in HIV-positive patients, the differences in these T cell subsets were not significant between HVL and LVL.

As the number of monocytes seemed to affect T cell stimulation by PHA, we also analyzed monocytes ($CD14^+$ cells) and found there was no quantitative difference between any of the groups (data not shown).

As the cytokine productive capacity of T cells differs according to their differentiation status,[25] we explored the differentiation status of $CD4^+$ and $CD8^+$ T cells. We divided $CD4^+$ and $CD8^+$ T cells into four subsets depending on the expression pattern of CD45RA and CCR7: naive ($CD45RA^+/CCR7^+$), central memory (CM; $CD45RA^-/CCR7^+$), effector memory (EM; $CD45RA^-/CCR7^-$), and effector ($CD45RA^+/CCR7^-$) subsets. The proportion of each subset was highly heterogenic between subjects. The HVL and LVL subjects showed no significant differences in distribution of T cell subsets except in the proportion of naive $CD8^+$ T cells (data not shown), which cannot secrete cytokines even following PHA stimuli (Fig. 3A).[26] ◀F3

To investigate whether there are qualitative differences in T cells between HVL and LVL subjects, we analyzed the expression of CD38, Ki67, Bcl2, PD-1, and CTLA-4 as markers of the activation and exhaustion status of T cells, which seems to affect their capacity to produce cytokines (Fig. 3B). In both $CD4^+$ and $CD8^+$ T cells, CM and EM subsets that mainly secrete these cytokines were highly activated ($CD38^+$, $Ki67^+$, and/or $Bcl\text{-}2^-$) in HVL subjects compared to LVL subjects (Fig. 3C). Especially in CM subsets of $CD4^+$ T cells, the frequency of exhausted cells ($PD\text{-}1^+$ and $CTLA\text{-}4^+$) was also significantly higher in HVL subjects compared to LVL subjects ($p < 0.05$ for both comparisons). EM subsets in $CD4^+$ T cells and CM and EM subsets in $CD8^+$ T cells also tended to be highly exhausted, although these differences were statistically insignificant in HVL subjects. These data indicate that memory $CD4^+$ and $CD8^+$ T cells, but not naive and effector subsets, are highly activated and exhausted in HVL subjects.

Poor cytokine production is directly correlated with activation/exhaustion status in memory T cells

As exhausted memory $CD8^+$ T cells fail to produce effector cytokines, such as IL-2, IFN-γ, and TNF-α, upon antigen stimulation,[27,28] we analyzed the relationship between the expression level of activation/exhaustion markers (CD38, Ki67, Bcl2, PD-1, and CTLA-4) on memory $CD4^+$ and $CD8^+$ T cells and the reduced production of cytokines seen in HVL subjects in response to PHA stimulation. The proportions of $PD\text{-}1^+$ and $CD38^+$ cells in CM subsets were inversely correlated with the capacity to produce MIP-1α, MIP-1β, RANTES, IFN-γ, and IL-17 (Fig. 4, and data not shown). In the EM subsets, proportions of $PD\text{-}1^+$ and $CD38^+$ cells, but not of $CTLA4^+$ cells, were inversely correlated with cytokine production. These data suggest that the compromised productive capacity of Th1-related and IL-17 cytokines is directly associated with persistent activation and exhaustion in memory T cells. ◀F4

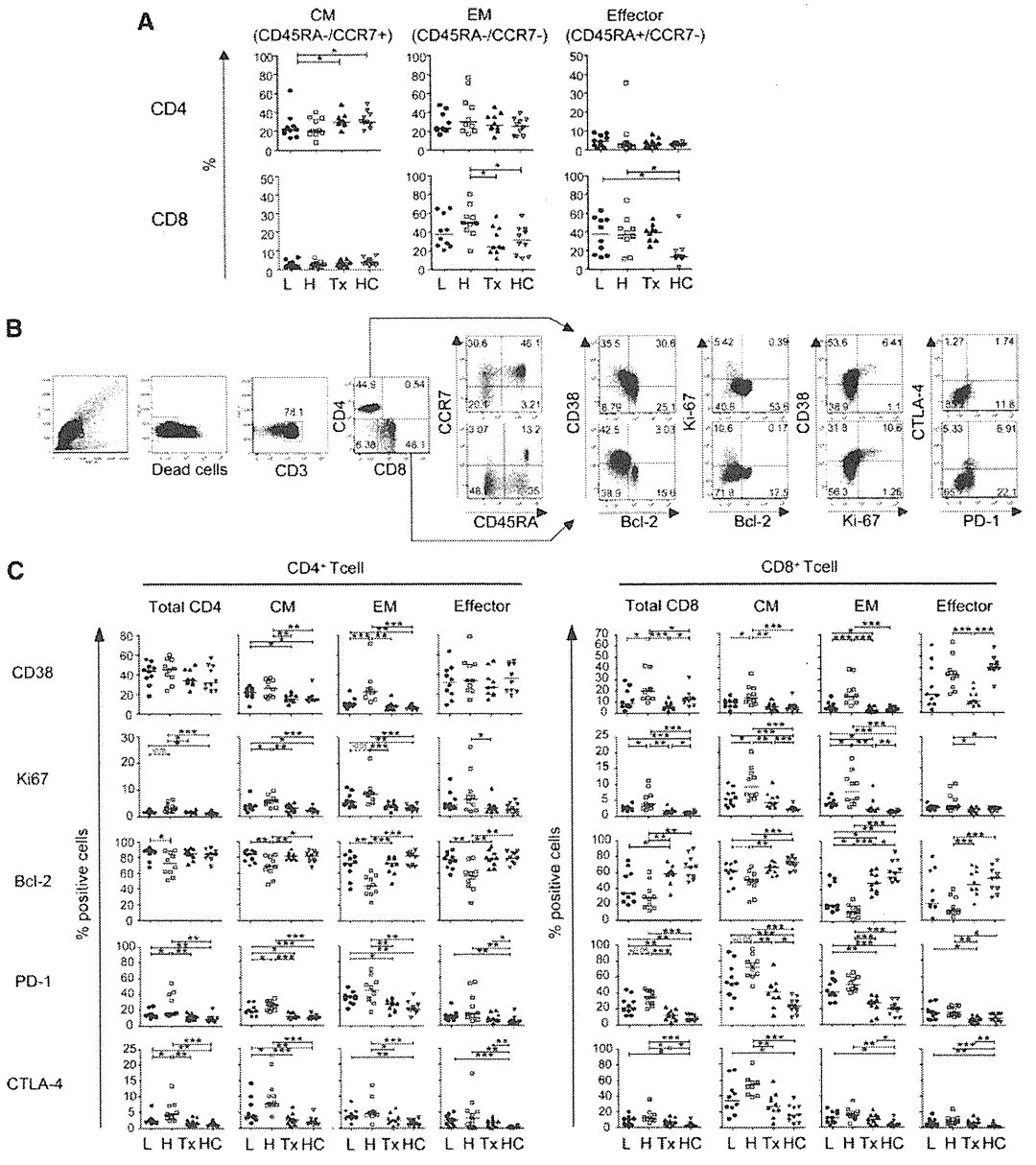
Cytokine production capacity is recovered soon after ART initiation, but memory CD8⁺ T cells remain activated and exhausted even after prolonged viral suppression by ART

To explore whether the low cytokine production in HVL subjects is a cause or a consequence of high viral load, we compared cytokine production in subjects whose VL had been suppressed by ART for a prolonged period (>22 months) and whose CD4 count was at a similar level to that of HVL and LVL subjects (Tx subjects). In these subjects, production of the cytokines that were decreased in HVL subjects (MIP-1α, MIP-1β, RANTES, IFN-γ, sIL-2R, IL-7, and IL-17) was significantly higher than in HVL subjects, and production of MIP-1α, MIP-1β, sIL-2R, IL-7, and IFN-γ was at a similar level to that seen in LVL and HC subjects (Fig. 1A). Production of RANTES and IL-17 was higher in subjects with long-term viral suppression than in the other groups.

To clarify the relationship between VL and cytokine production capacity, we performed a similar analysis in subjects with dramatic reductions in VL due to recent ART initiation. We measured cytokine production from PBMCs isolated from blood drawn from six HIV-1-infected subjects within 1–2 months after starting ART, when VL had undergone dramatic reduction (mean VL = 440 copies/ml, range 63 to 1100) (Fig. 5A). The levels of cytokines MIP-1α, MIP-1β, RANTES, and IL-7 produced after PHA stimulation were comparable to those seen in subjects with long-term suppression from ART (Fig. 5B). These data indicate that dysfunction of these cytokine production in individuals with high VL is reversible and is recovered soon after the VL reduction. ◀F5

We also analyzed the activation and exhaustion status of $CD4^+$ and $CD8^+$ T cells in treatment-experienced (Tx) subjects (Fig. 3C). With the sole exception of CTLA-4 expression on the EM subset in $CD8^+$ T cells, proportions of activated ($CD38^+$, $Ki67^+$, and $Bcl\text{-}2^-$) cells and exhausted ($PD\text{-}1^+$ and $CTLA\text{-}4^+$) cells within both memory $CD4^+$ and $CD8^+$ T cell populations were significantly lower in Tx subjects compared to HVL subjects (Fig. 3C).

We next examined the activation/exhaustion status of memory $CD4^+$ and $CD8^+$ cells in Tx subjects compared to uninfected control subjects to determine whether the T cell status can revert to normal status after prolonged viral suppression by ART. The Tx and HC groups did not differ significantly in expression levels of markers in memory $CD4^+$ T cells (Fig. 3C). In contrast, the two groups differed significant in the activation/exhaustion status of memory $CD8^+$ T cells, with higher levels of Ki67, PD-1, and/or CTLA-4 expression and lower levels of Bcl-2 expression, in Tx subjects compared



AU3 ▶ **FIG. 3.** Differentiation and activation/exhaustion status of $CD4^+$ and $CD8^+$ T cells in HIV-1-infected subjects. (**A**) Comparison of the frequency of $CD4^+$ and $CD8^+$ T cell subsets. The percentages of central memory (CM; $CD45RA^-/CCR7^+$), effector memory (EM; $CD45RA^-/CCR7^-$), and effector ($CD45RA^+/CCR7^-$) subsets in $CD4^+$ and $CD8^+$ T cells are shown. (**B**) Representative flow cytometric analysis of activation (CD38, Ki67, and Bcl2 expression) and exhaustion (PD-1 and CTLA-4 expression) status in $CD4^+$ and $CD8^+$ T cells. (**C**) Comparison of the activation/exhaustion status in $CD4^+$ and $CD8^+$ T cell subsets. L, LVL subjects (●), H, HVL subjects (□), Tx, HIV-1-infected subjects with prolonged ART (▲), HC, healthy control (□). The horizontal bars indicate the median value. Differences between groups were tested for statistical significance by the Mann–Whitney *U* test. $*p = 0.01$ to 0.05, $**p = 0.001$ to 0.01, $***p < 0.001$ (Mann–Whitney test).

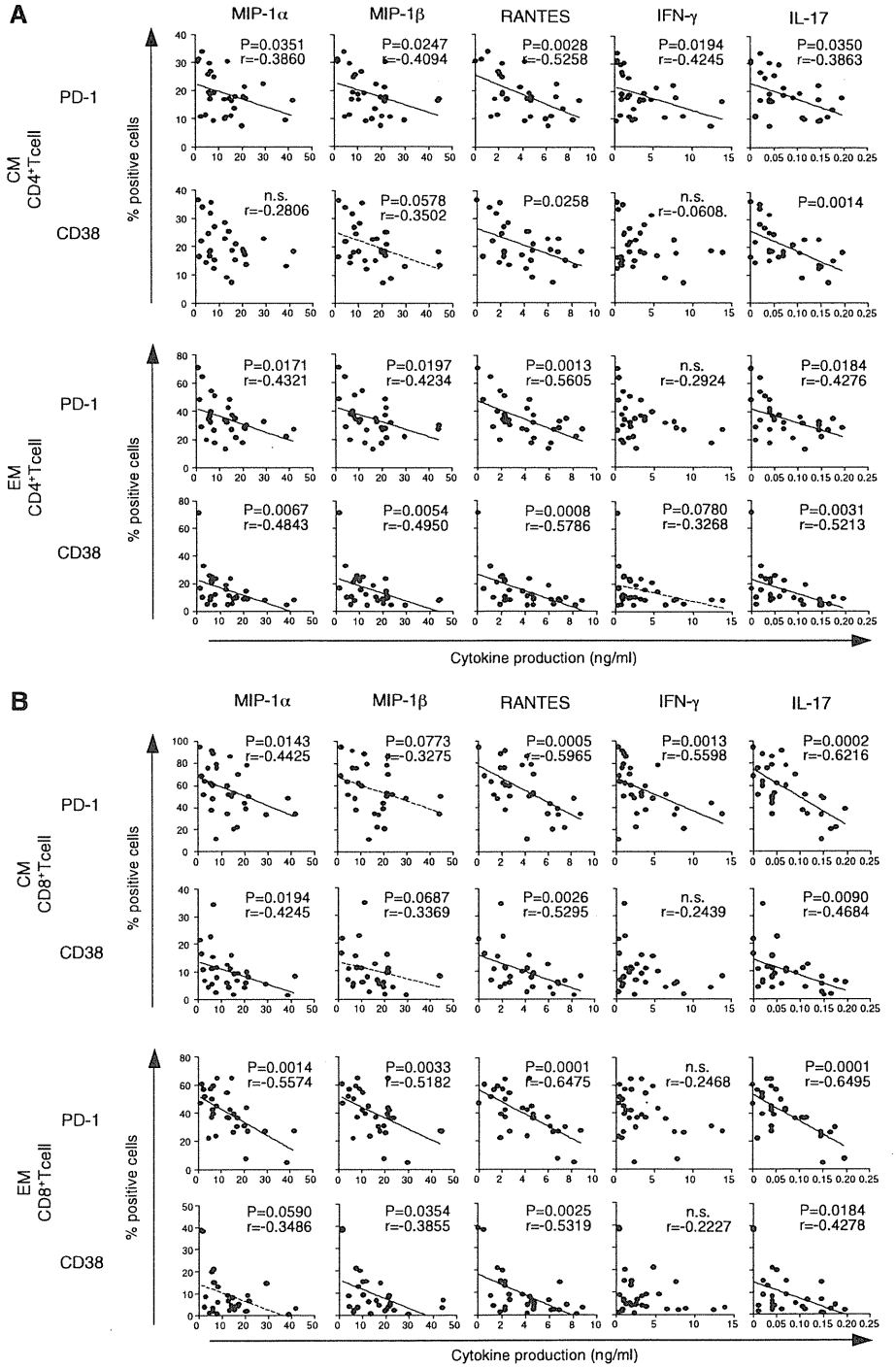


FIG. 4. Correlation between activation/exhaustion status in memory T cell subsets and cytokine production. Each panel indicates the relationship between the frequency of PD-1 or CD38 expressing cells in central memory (CM) and effector memory (EM) T cells and each cytokine production. The results of $CD4^+$ T cells and $CD8^+$ T cells are shown in (**A**) and (**B**), respectively. Correlation analysis was performed with Spearman's rank correlation to determine correlations between variables.

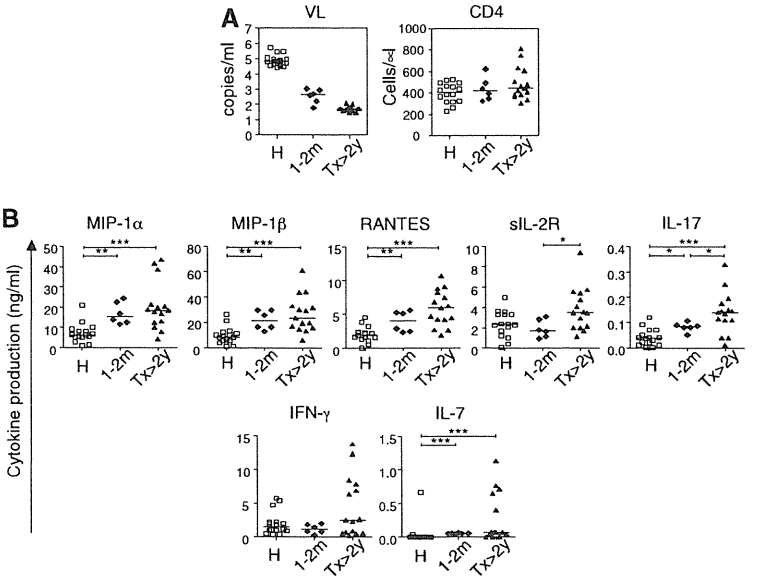


FIG. 5. Rapid recovery of cytokine production after initiation of antiretroviral therapy. **(A)** Viral load and CD4 count of each group. **(B)** Comparison of cytokine production by PHA-stimulated PBMCs. H, HVL subjects (□), 1–2 months, subjects 1–2 months after starting ART (□), Tx>2 years, subjects with prolonged ART (▲). $^{*}p=0.01$ to 0.05, $^{**}p=0.001$ to 0.01, $^{***}p<0.001$ (Mann–Whitney test).

to HC subjects. These data suggest that although suppression of HIV-1 replication by ART dramatically improves the cytokine production capacity of T cells to a normal level, memory $CD8^+$ T cells, but not memory $CD4^+$ T cells, remain somewhat activated even after prolonged viral suppression.

Discussion

Despite intensive research, it remains unclear how HIV-1 can cause the collapse of the host immune system and development of AIDS after chronic infection. In this study, we demonstrate that high HIV-1 viral load associates with skewed T cell dysfunction in cytokine production, independently of CD4 T cell count. Diminished cytokine production in subjects with high VL is specific for some Th1-related cytokines (MIP1-α, MIP-1β, RANTES, and IFN-γ), IL-17, IL-7, and sIL-2R, and is associated with activation and exhaustion status in both $CD4^+$ and $CD8^+$ T cells, especially in memory subsets. The dysfunctional production of these cytokines in HVL subjects appears to be reversible, with recovery occurring after VL reduction by ART.

In this study, we tried to find as many cytokines as possible that differ between LVL and HVL subjects. For this reason, we used a strong stimulus and long incubation times to show the results clearly. The 48-h culture period is long enough to allow expression both of late-response genes and of secondary response genes that may be induced following the primary response.

Production of MIP-1α, MIP-1β, and RANTES was dramatically reduced in HVL subjects and showed a close inverse correlation with plasma VL (Fig. 1). As the natural ligands of HIV-1 coreceptor CCR5, MIP-1α/MIP-1β/RANTES are potent inhibitors of CCR5-tropic HIV-1 (R5-HIV-1) infection.[29] Physiologically, these chemokines also play a key role in induction of cellular immune responses by recruiting $CCR5^+$ Th1 lymphocytes to the infectious site *in vivo*.[30–33] In the case of HIV-1 infection, decreased production of these chemokines seems to favor both viral expansion and reduced migration of effector T cells *in vivo*. In recent studies, a high copy number of CCL3L1 (one of the genes encoding MIP-1α) combined with a low CCR5 expression genotype was associated with low VL in HIV-1-infected subjects,[34,35] suggesting that CCL3L1-CCR5 genotypes may be able to modify the clinical course of HIV-1 infection.

In our study plasma VL affected the ability of T cells to produce IFN-γ, one of the cytokines that defines Th1 cells, and IL-17, which is a Th17-type cytokine. However, no effect was seen on Th2-type cytokines (IL-4, IL-5, and IL-13) or IL-10. Interferon-γ, MIP-1α, MIP-1β,and RANTES are produced by Th1 cells (Fig. 2B), which preferentially express CCR5,[30] and Th17 cells are known to express CCR5 in peripheral blood.[36,37] However, Th2 cells do not express CCR5.[30] Transcription of these cytokines in T cells may be influenced by CCR5 signaling. Large amounts of R5-HIV-1 or the Env protein might persistently trigger the signaling pathway by binding to CCR5, thereby causing reductions in levels of specific cytokines in chronically HIV-1 subjects.

In our experiments, MIP-1α, MIP-1β, and RANTES were produced by IFN-γ-expressing cells in subsets of $CD4^+$ and $CD8^+$ T cells, and IL-17 was produced by a different subset of $CD4^+$ T cells. Surprisingly, IL-17 production was strongly correlated with MIP-1α, MIP-1β, RANTES, and IFN-γ production even though the producer cells are different (Fig. 2F). This correlation might reflect a general ability of Th1 and Th17 cells to produce cytokines. However, IL-2 production was not correlated with MIP-1α, MIP-1β, RANTES, and IFN-γ production, despite the fact that IL-2 should be produced by the same IFN-γ-producing cells (Fig. 2C–F). Critical regions of IFN-γ promoter (i.e., consensus GATA motif and essential functional motif) are not found in the IL-2 promoter region, but are found in the MIP-1α and MIP-1β promoters.[38] In addition, the same sequence in the promoter region of IFN-γ,

MIP-1α, and MIP-1β was found in the IL-17 promoter.[39] Interferon-γ, MIP-1α, MIP-1β, RANTES, and IL-17 production in T cells may be coordinately regulated, and the productive capacity of these cytokines appears to be affected by HIV-1 VL in a similar fashion. Alternatively, we measured cytokine production 48 h after PHA stimulation in this study. The period is long enough to develop sequential reactions occurring in response to primary reaction. As IFN-γ is known as an early-response gene and has the potential to affect multiple immune responses,[40] the production of MIP-1α, MIP-1β, RANTES, and IL-17, but not IL-2 may depend on the amount of IFN-γ as the primary response. Further studies are required to elucidate the mechanism by which IL-17 production is correlated with MIP-1α, MIP-1β, RANTES, or IFN-γ production. The IFN-γ pathway protects against intracellular pathogens through cellular immunity, and IL-17 provides protection against extracellular pathogens and fungal infections.[41,42] Although their target pathogens differ, IL-17 regulates the Th1 immune response through IL-17 receptor-expressing dendritic cells (DC) and macrophages.[43] These data suggest that Th1-type and Th17-type immune responses are closely related, and that their interaction is crucial for immune protection.

In a pathogenic SIV infection model, the loss of Th17 cells in the gastrointestinal tract dampens the intestinal mucosal barrier, resulting in microbial translocation, which in turn induces systemic immune activation.[44–47] In SIV infection the loss of Th17 cells in intestinal mucosa and in PBMCs is inversely correlated with plasma VL.[48] In this study, we observed a strong inverse correlation between IL-17 production and the proportion of activated and exhausted memory T cells. Our results suggest that not only the number of IL-17-producing cells but also the quality of those cells may account for the dysfunction of the Th17-type immune response in HVL subjects.

During chronic HIV-1 infection expression of the inhibitory coreceptors PD-1 and CTLA-4 on total T cells (not only HIV-1-specific T cells) is associated with plasma VL and CD4 count.[11,12] In this study, we found that the proportions of PD-1$^+$, CTLA4$^+$, and CD38$^+$ cells in total memory subsets of CD4$^+$ and CD8$^+$ T cells were inversely correlated with the ability of T cells to produce MIP-1α, MIP-1β, RANTES, IFN-γ, sIL-2R, and IL-17 in response to PHA stimulation (Fig. 4). It has been reported that PD-1 expression depends on the status of activation markers such as CD38 and on the differentiation stage of T cells.[49,50] Other studies have shown that blocking the pathway of the PD-1/PD-L1 interaction augments the cytokine production capacity of HIV-1-specific CD4$^+$ and CD8$^+$ T cells *in vitro*.[11,51] In our study, prolonged virus suppression by ART resulted in cytokine production capacities returning to normal (Fig. 1A). Memory subsets of CD4$^+$ T cells were no longer activated and exhausted (Fig. 3C), although memory subsets of CD8$^+$ T cells remained slightly activated/exhausted. These data suggest that activation and/or exhaustion of T cells is directly associated with the ability to produce these specific cytokines and that the impairment in T cell function is reversible.

Our study is the first to show that the T cell impairment in high VL subjects is specific for production of some of Th1-type and Th17-type cytokines, and that production of these cytokines is strongly correlated with one another. In subjects with high VL, a vicious cycle occurs, as T cells increasingly lose the capacity to produce these important cytokines. Notably, we also found that subjects who maintain a low VL, yet who are not "elite controllers," are capable of producing normal levels of these cytokines. These findings could be useful in guiding the development of new therapies focusing on immune control to reduce T cell activation in chronic HIV-1 infection.

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Author Disclosure Statement

No competing financial interests exist.

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Unique CRF01_AE Gag CTL Epitopes Associated with Lower HIV-Viral Load and Delayed Disease Progression in a Cohort of HIV-Infected Thais

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Abstract

Cytotoxic T Lymphocytes (CTLs) play a central role in controlling HIV-replication. Although numerous CTL epitopes have been described, most are in subtype B or C infection. Little is known about CTL responses in CRF01_AE infection. Gag CTL responses were investigated in a cohort of 137 treatment-naïve HIV-1 infected Thai patients with high CD4+ T cell counts, using gIFN Enzyme-Linked Immunospot (ELISpot) assays with 15-mer overlapping peptides (OLPs) derived from locally dominant CRF01_AE Gag sequences. 44 OLPs were recognized in 112 (81.8%) individuals. Both the breadth and magnitude of the CTL response, particularly against the p24 region, positively correlated with CD4+ T cell count and inversely correlated with HIV viral load. The breadth of OLP response was also associated with slower progression to antiretroviral therapy initiation. Statistical analysis and single peptide ELISpot assay identified at least 17 significant associations between reactive OLP and HLA in 12 OLP regions; 6 OLP-HLA associations (35.3%) were not compatible with previously reported CTL epitopes, suggesting that these contained new CTL Gag epitopes. A substantial proportion of CTL epitopes in CRF01_AE infection differ from subtype B or C. However, the pattern of protective CTL responses is similar; Gag CTL responses, particularly against p24, control viral replication and slow clinical progression.

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Introduction

Cytotoxic T-Lymphocytes (CTLs) are an important component of the adaptive immune system which mediate control of HIV replication during acute infection and consequent viral set point [1]. Numerous CTL epitopes have been reported across the HIV proteome. However, the influence of CTL on clinical outcome varies, as their recognition of viral antigen is restricted by highly polymorphic class I Human Leukocyte Antigen (HLA) molecules [2,3]. Furthermore, the tremendous degree of viral diversity increases this complexity; to date, 13 prototype HIV clades and 43 circulating recombinant forms (CRF) have been described [4]. Some epitopes have been reported in a single clade; others have been reported in multiple clades (cross-clade) [5,6]. No reported epitope to date universally covers all HIV subtypes, or overcomes the global variation in HLA allele distribution (CTL Epitopes. Los Alamos National Lab. http://www.hiv.lanl.gov/).

Gag CTL responses, but not other CTL responses, have consistently been reported to have a significant association with viral control and clinical outcome [7]. However these findings were derived mainly from African or Caucasian populations infected with subtype C or B HIV, respectively; data from Asian populations infected with subtypes circulating in south-east Asia, such as CRF01_AE, have not yet been reported. To determine whether a similar association exists in south-east Asian subtypes, CTL epitope information is essential. However, as of April 2011, only 26 of 420 known Gag epitopes have been reported in CRF01_AE infection. Recently, the first successful phase III HIV vaccine trial was reported from Thailand [8], although its efficacy was marginal. For the development of a more effective vaccine, we believe it is crucial to accurately understand the influence of sequence variation amongst HIV subtypes, and HLA diversity amongst ethnic groups. To provide more information about CTL epitopes in CRF01_AE infection, we investigated cellular immune responses to Gag overlapping peptides in an HIV-1 CRF01_AE-infected Thai population and evaluated their impact on clinical outcome.

Methods

Subjects

This study was approved by the Thai Ministry of Public Health Ethics Committee and was conducted according to set guidelines for research. Written informed consent was obtained after

explaining the purpose and expected consequences of the study. Patients were eligible for inclusion if they were chronically HIV-infected and antiretroviral-naïve, with a CD4+ T cell count >200 cells/ul. A total of 137 HIV-1 CRF01_AE infected individuals were recruited at a government referral hospital in Thailand from October 2003 to May 2009. Study subjects were requested to visit the clinic every 3 months and CTL responses were evaluated every 6 months. The study endpoint was initiation of antiretroviral therapy, when their CD4+ T cell count declined below 200 cells/ul.

Synthetic HIV-1 Gag overlapping peptides

Fifteen-mer overlapping peptides (OLPs) of locally dominant CRF01_AE Gag sequences were designed based on 125 *gag* clonal sequences derived from 45 CRF01_AE infected individuals attending the clinic. All deduced amino-acid sequence data were aligned and the most frequent 15-mer amino-acid sequence was used as the dominant sequence.

Peptides were synthesized by Sigma Genosys (Hokkaido, Japan) with a high purity of >90% as determined by high-pressure liquid chromatography. In total, 98 peptides were synthesized and 20 pools were made by mixing 10 peptides per pool in a 10×10 matrix design so that a single responsible peptide could be identified by detecting the common peptide between two reactive pools, as described previously [9–11]. When more than one peptide was recognized, we further confirmed the responsible peptide recognition by individually testing candidate peptides, which were suspected by the matrix method.

ELISpot assay

1×10^5 fresh PBMC/well were plated onto multiScreen plates (MAHA54510; Millipore) that had been coated overnight at 4°C with 50 µl of anti-gIFN capture Ab 1-D1-K (2 µg/ml; Mabtech, Ohio, USA). Peptides were added directly to wells at a final concentration of 1 µM in 50 µl of R10 and incubated at 37°C in 5% CO_2 for 24 hrs. PBMC were stimulated with either medium alone for negative control, 10 µg/ml phytohemagglutinin (PHA; Sigma-Aldrich) for positive control or peptide (1 µM final concentration) for 24 hrs at 37°C. Plates were washed extensively with wash buffer (PBS/Tween20 0.001%), followed by incubation with biotinylated anti-human gIFN mAb (0.5 µg/ml; clone 7-B6-1; Mabtech) in PBS/10% FBS for 2 hrs at 37°C. Following six further washes with wash buffer, 2 µg/ml streptavidin HRP (Mabtech) was added to wells with 1 hr incubation at room temperature. Spots were visualized using BCIP/NBT substrate (Chemicon, Australia) and were counted using an Automated Enzyme-Linked Immunospot (ELISpot) Reader System with KS 4.3 software by an independent scientist in a blinded fashion. Each assay was undertaken in triplicate. Spot forming units (SFU) were counted and expressed as SFU per million PBMCs, using the average result from triplicate wells followed by subtraction of the negative control values. A response was defined as positive if it was three times higher than the negative control and greater than 150 SFU/1×10^6 PBMC. The breadth of response was defined as the total number of peptides recognized by each subject. The magnitude of response for an individual was defined as the sum of all positive peptide responses (in SFU/1×10^6 PBMC). To avoid overestimation of breadth or magnitude, two adjacent positive overlapping peptides were counted as one response, using the higher of the two responses.

HLA class I typing

Genomic DNA was extracted from buffy coat using the QIAamp DNA blood Mini Kit (Qiagen, Hilden, Germany) and 4-digit HLA class I typing for A, B and Cw loci was undertaken by bead-based array hybridization (WAKFlow HLA typing kit, Wakunaga Pharmaceutical, Hiroshima, Japan) according to manufacturer's instructions at a commercial laboratory (Kyoto HLA Laboratory, Kyoto, Japan).

Statistical analysis

Statistical analysis was performed using EXCEL 2007 and SPSS. We first selected viral loads (VL) in the lowest (=q1) and highest (=q4) quartiles (n=34 for each) and compared the number of individuals with positive ELISpot responses to p17, p24 and p15 proteins, using Fisher's exact test to compare groups. We then analyzed the association between breadth and clinical outcome (CD4+ T cell count and VL), using the Kruskal-Wallis test, and between magnitude and clinical outcome (CD4+ T cell count and VL) using Spearman's correlation test. We also performed a longitudinal analysis of the effect of breadth on Highly Active Anti-Retroviral Therapy (HAART) initiation, using the log rank test and Cox regression. For this analysis, the first individual was enrolled on 6 July 2000 and the last individual on 4 September 2007, with a censoring date of 31 May 2009. Analysis of OLP-HLA associations was undertaken using Fisher's exact test with 95% confidential intervals (CI). To have enough statistical power, we analyzed OLP-HLA associations when OLPs were recognized by 3 or more individuals with relevant HLA alleles and at least in one individual, the OLP recognition was confirmed by single peptide ELISpot experiments.

Results

Individuals' background, including HLA distribution

Of 137 individuals recruited, 107 were female and 30 were male. Median age was 31 years (range 16–56), CD4+ T cell count 461 cells/ul (range 204–1,191), and VL 4.22 log copies/ml (range 2.60–5.88). No individual had any HIV-related symptoms at the time of enrollment. In total, 87 variations of HLA alleles were found: 23 variations in HLA_A, 46 in HLA_B and 18 in HLA_Cw in four digits (Table S1). Median duration of follow-up was 22 months (range 0–60) and ELISpot experiments were repeated median 4 times (range 1–11) per individual. The peptide recognition pattern was confirmed to be consistent on at least two occasions for all except 24 individuals, in whom ELISpot assays were undertaken only once. During the follow-up period, the peptide recognition pattern did not change in any individual.

Gag OLP recognition and clinical outcome

Among 137 individuals, 112 (81.8%) recognized at least one OLP. Of 98 OLPs, 44 (44.9%) were recognized by at least one individual (Figure 1A): 12 peptides in p17, 26 in p24 and 6 in p15. The second half of p24 (HXB2 261–360; OLP 52–69), was the most highly targeted protein region; the first half of p17 (HXB2 5–60; OLP 1–9) was the second most highly targeted region. 14 OLPs were recognized in one individual and the other 30 OLPs were recognized in more than one individual. The most frequently recognized peptides were all located in the second half of p24: OLP 54 (HXB2 271–285), was recognized by 27 individuals; OLP 59 (HXB2 296–310) by 23 individuals; and OLP 66 (HXB2 331–345) by 22 individuals.

To further elucidate the peptide recognition pattern that best contributes to viral control, we next compared ELISpot responses between two extreme VL groups: the lowest quartile (=q1) (median VL 3.27 log copies/ml (range 2.60–3.71)) and the highest quartile (=q4) (median 5.09 log copies/ml (range 4.76–5.88)) (Figure 1B). Median CD4+ T cell count was 515 cells/ul (range

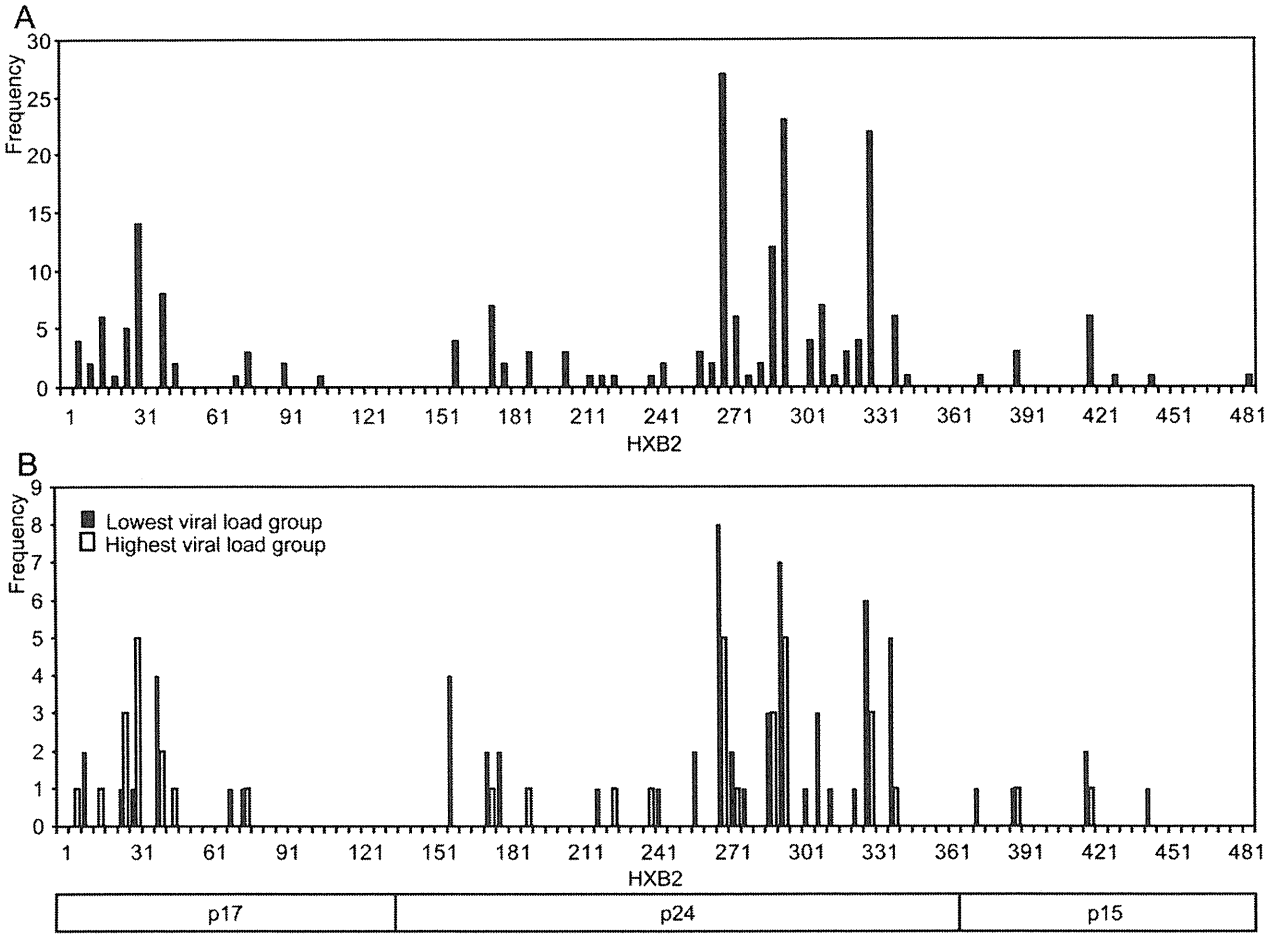

Figure 1. Pattern of CRF01_AE Gag CTL responses. Frequencies of overlapping peptide (OLP) responses in 112 individuals are shown (A); Frequencies of OLP responses in the lowest viral load group (lowest quartile, n = 34) and the highest viral load group (highest quartile, n = 34) were compared (B).
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243–1,057) in q1 and 429 cells/ul (range 204–856) in q4 (p = 0.022). Interestingly, individuals in q1 more frequently recognized p24 peptides than those in q4 (29/34 vs 18/34, respectively; p = 0.0018, Fisher's exact test), whereas individuals in q4 tended to recognize p17 peptides more frequently (9/34 vs 12/34, respectively; p = 0.6), although this difference was not significant.

ELISpot breadth, magnitude and clinical outcome

We next investigated the relationship between breadth and clinical outcome. The CD4+ T cell count was significantly higher in individuals with a greater breadth of response, with median CD4+ T cell count of 409 cells/ul (range 204–995), 455 cells/ul (range 243–793), 495 cells/ul (range 264–1,087) and 538 cells/ul (range 303–1,191) in individuals with 0, 1, 2 and ≥3 responses, respectively (p = 0.018 by Kruskal-Wallis test) (Figure 2A left). VL was significantly lower in individuals with a greater breadth of response, with median VL of 4.83 log copies/ml (range 2.60–5.88), 4.21 log copies/ml (range 2.60–5.83), 4.26 log copies/ml (range 2.76–5.71) and 3.82 log copies/ml (range 2.60–5.04) in individuals with 0, 1, 2 and ≥3 responses, respectively (p = 0.0015) (Figure 2A right). In a site-specific analysis, we did not find any significant association with CD4+ T cell count in any sites (Figure 2B). Interestingly, we found a significant association with VL only in p24 (4.57 log copies/ml (range 2.60–5.88), 4.21 log copies/ml (range 2.60–5.80), 4.17 log copies/ml (range 2.60–5.23) and 3.37 log copies/ml (range 2.60–4.14) in individuals with 0, 1, 2 and ≥3 responses, respectively; p = 0.00028) but not in other sites (Figure 2C).

We also found that magnitude of ELISpot response was positively correlated with CD4+ T cell count (p = 0.0032 by Spearman's correlation test $y = 0.031x+453$ $R^2 = 0.080$) and inversely correlated with VL (p = 0.0084 $y = -0.0001x+4.41$ $R^2 = 0.055$) (Figure 3A). In a detailed site-specific analysis, magnitude in p24 had a significant correlation with clinical outcome both in CD4+ T cell count (p = 0.048 $y = 0.013x+493$ $R^2 = 0.010$) (Figure 3B) and VL (p = 0.0018 $y = -0.0001x+4.39$ $R^2 = 0.065$) (Figure 3C), but not in other sites.

We next investigated the effect of breadth on clinical progression using the initiation of antiretroviral therapy as the end-point. During the follow-up period, 66/137 (48.2%) individuals started antiretroviral therapy. Intriguingly, we found that individuals with a wider breadth of CTL response were less likely to start antiretroviral therapy than those with a narrower breadth of response (Figure 4A, p = 0.001 by log rank test): 18/25 (72.0%), 13/34 (38.2%), 30/57 (52.6%) and 5/21(23.8%) individuals with 0, 1, 2 and ≥3 responses, respectively, initiating antiretroviral therapy. These data imply that strong CTL responses delay

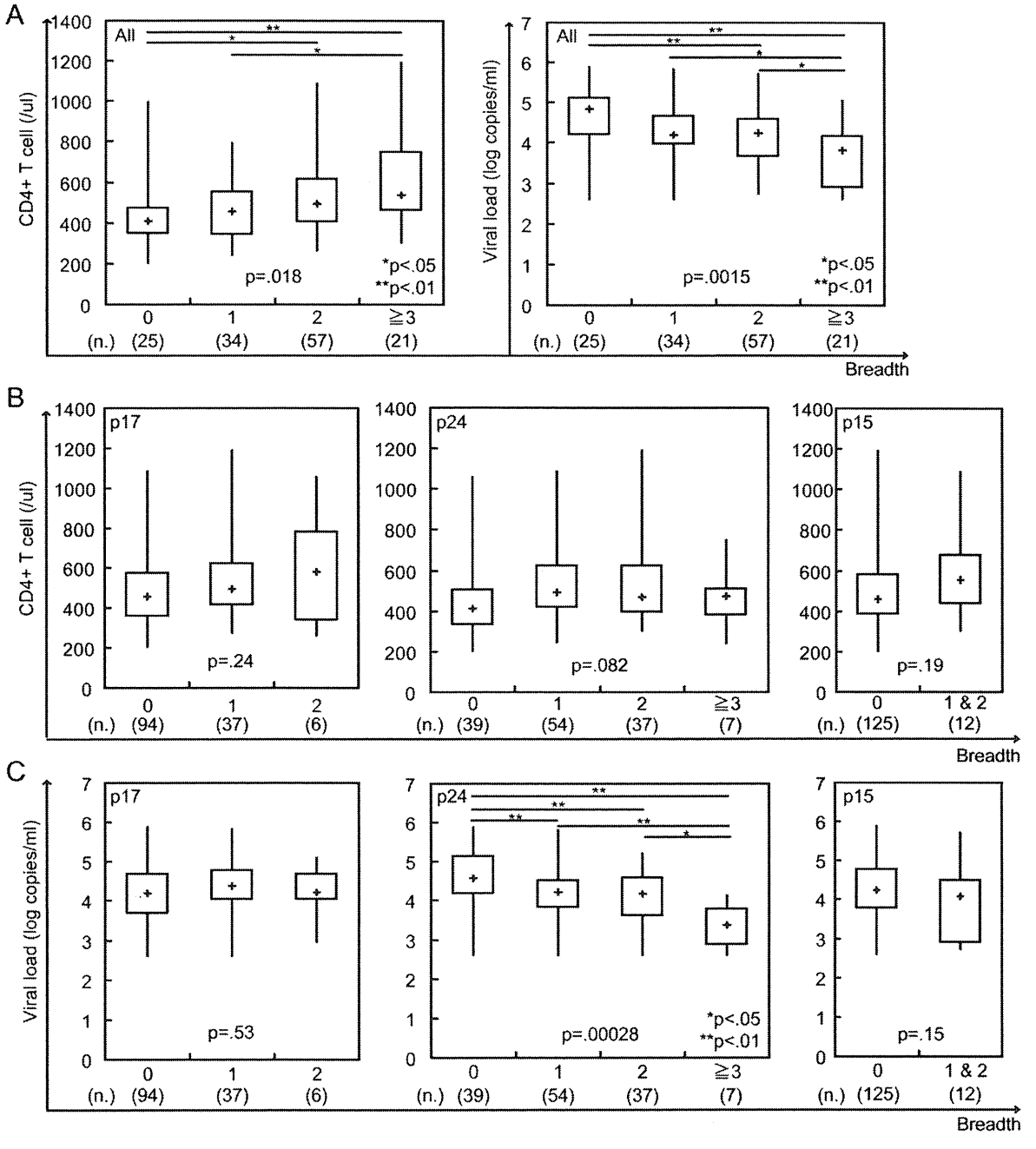

Figure 2. ELISpot breadth is associated with CD4+ T cell count and viral load. The associations between ELISpot breadth (the number of reacting OLP) and CD4+ T cell count or viral load were analyzed using the Kruskal-Wallis test (A). The p17, p24 or p15 site-specific ELISpot breadth was also compared with CD4+ T cell count (B) and viral load (C); * and ** showed a significant difference of $p<0.05$(*) and $p<0.01$(**) by Mann-Whitney u-test.

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clinical progression by slowing the decline in CD4+ T cell count. In a detailed site-specific analysis, individuals with a p24 response, but not other responses, were significantly less likely to start antiretroviral therapy than individuals without a p24 response ($p = 0.001$). However, the breadth of p24 response did not seem to correlate with clinical progression (Figure 4B).

Multivariate analysis of the relationship between CTL response and initiation of antiretroviral therapy, using Cox proportional hazards model, showed that the association between breadth of CTL response and initiation of HAART was independent of the baseline CD4+ T cell count (>350 cells/ul or not) and VL (<4.0 log copies/ml, 4.0–4.9 log copies/ml and ≥5.0 log copies/ml): adjusted Hazard Ratio (aHR) for individuals making ≥3 OLP responses was 0.23 ($p = 0.005$ with 95% CI of 0.08–0.64).

Detection of reactive OLP-HLA association

Associations between OLP responses and HLA were statistically analyzed. In total, 14 peptides (4 in p17, 9 in p24 and 1 in p15) with 31 OLP-HLA associations were identified (Table S2). 13 associations were found both with HLA-B and Cw alleles each and

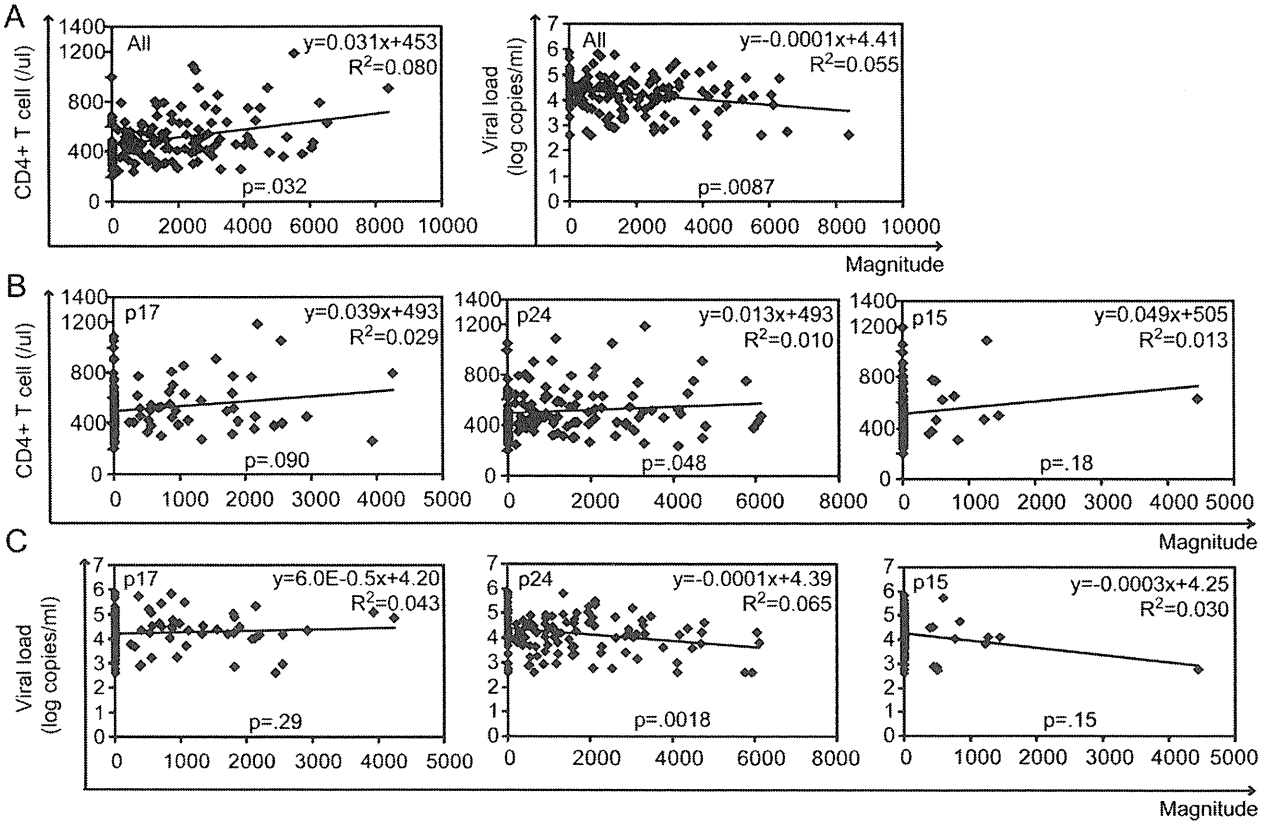

Figure 3. ELISpot magnitude is associated with CD4+ T cell count and viral load. The associations between ELISpot magnitude (total SFU per 1.0 M PBMC) and CD4+ T cell count or viral load were analyzed by Spearman's correlation (A). The p17, p24 or p15 site-specific response was also compared with CD4+ T cell count (B) and viral load (C).
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5 were found with HLA-A alleles. Two adjacent OLPs shared the same responsible HLA allele: HLA_A*0207, B*4601 and Cw*0102 in OLP 54–55, and B*4601 in OLP 58–59, suggesting that CTL epitopes reside in the overlapping region of these peptides. Some of the OLP-HLA associations may not reflect genuine CTL epitopes. 10 OLP responses were associated with two or more responsible HLA alleles. Of these, 9 OLP responses were associated with a pair of HLA alleles in linkage disequilibrium (LD), which were identified using the Los Alamos database (HLA Linkage Disequilibrium. Los Alamos National Lab. http://www.hiv.lanl.gov/). Among the 10 OLP responses, 7 included reported epitopes in either one of the HLA alleles. OLP 54, 55 and 59 responses were also associated with HLA alleles that have haplotype associations: HLA_A*0207-B*4601-Cw*0102. In total, 11 OLP-HLA associations were compatible with previously reported CTL epitopes: 4 epitopes were already reported as cross-clade epitopes including CRF01_AE or subtype A and the remaining 7 epitopes were reported in other subtypes but neither in subtype A nor CRF01_AE. Consequently, we identified at least 17 OLP-HLA associations in 12 OLP regions; 6 OLP-HLA associations (35.3%) were not compatible with previously reported CTL epitopes, suggesting that these are likely to contain unique CRF01_AE Gag CTL epitopes.

Discussion

This is the first study to investigate Gag CTL epitopes and their effect on clinical outcome in a systematic way in a CRF01_AE-infected Asian cohort. In this study, which tested optimal OLPs in a well-described cohort, we succeeded in predicting a number of unique CRF01_AE Gag epitope and novel cross-clade epitope candidates. Although one third of CTL epitope candidates in CRF01_AE infection were not compatible with previously reported CTL epitopes in other subtypes, both cross-sectional and longitudinal analysis showed the pattern of protective CTL responses was similar to previous studies; specifically, that a Gag CTL response, particularly against p24, was associated with better control of viral replication and slower clinical progression [7,11–15]. These findings are also compatible with our previous study in which an association with clinical outcome was found only for the number of HLA-associated mutations in p24 but not in other sites [16]. Both studies imply that immune pressure on p24 Gag influences the clinical outcome in CRF01_AE infected Asian individuals. Several papers have discussed the advantages of CTL immune pressure against p24 for viral control , which include selection of escape mutations that lead to viral fitness cost [17,18], sequence stability compared with other viral particles [4,19,20], the abundance of Gag protein in incoming virions [21], and more rapid antigen presentation of Gag epitopes following viral infection [18].

While our findings showed the clear-cut relationship between ELISpot breadth and clinical parameters, the slopes of the trend lines between ELISpot magnitude and clinical parameters were rather shallow. Furthermore ELISpot magnitude did not correlate with onset of HAART initiation. These findings are consistent with a recently published study that breadth of the CTL response rather than magnitude associated best with clinical outcome [22].

In this study, we could not detect any OLP-HLA associations in HLA_B*57, which is well-known as one of the most protective

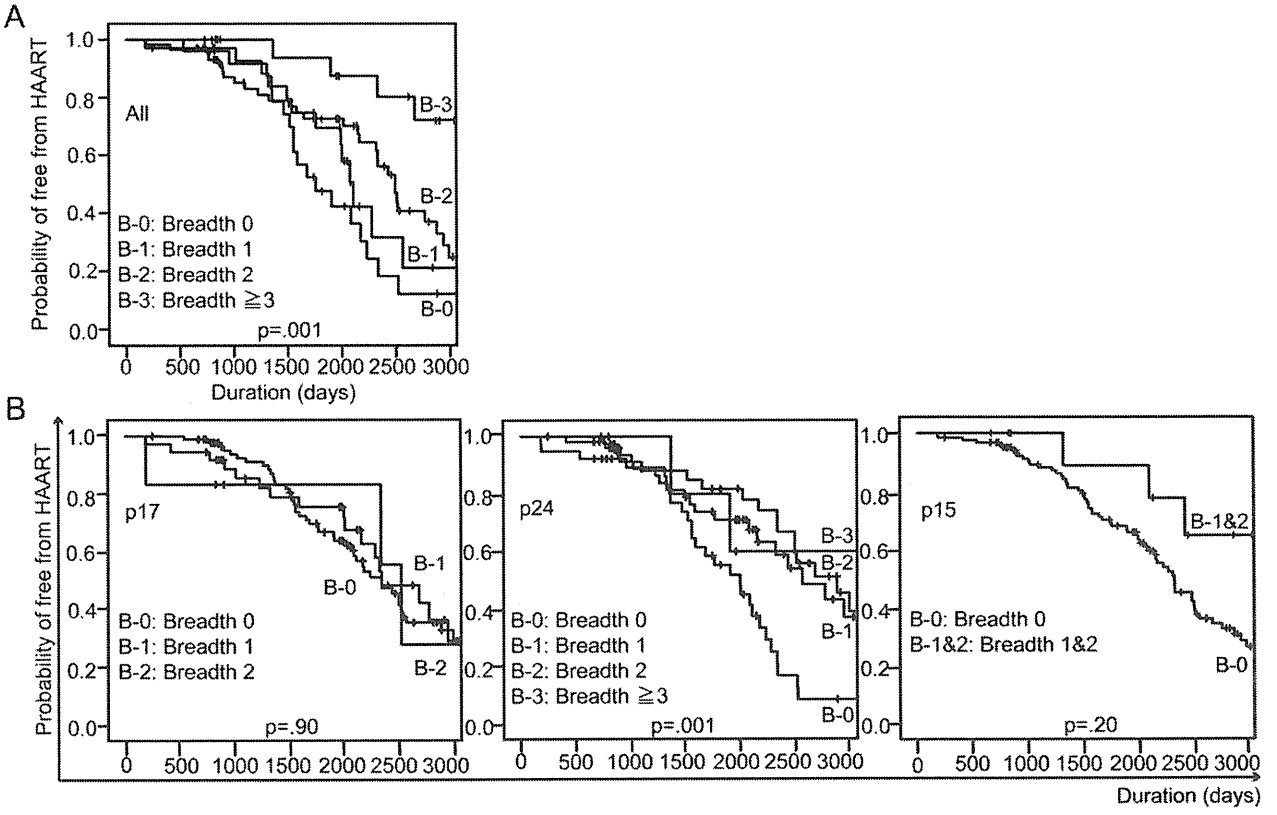

Figure 4. ELISpot breadth is related to delayed initiation of antiretroviral therapy. The impact of ELISpot breadth on antiretroviral therapy initiation was evaluated by Kaplan-Meier analysis, using the log rank test (A). The effect of p17, p24 or p15 site-specific ELISpot breadth was also analyzed (B).
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alleles for viral control [2,3,23]. Three individuals expressed B*5701; however, none had any response to OLP 47, which contains the TW10 (TSTLQEQIGW) epitope [24]. We have previously found in our cohort that all B*57 patients had the T242N escape mutation [16]. This suggests that the virus circulating in B*57 individuals lacks the wild-type TW10 sequence *in vivo* and no longer stimulates TW10 CTL cells [25].

In this study, OLP-HLA associations were predicted by statistical analysis. Thus these associations are not necessarily a reflection of new CTL epitopes with responsible HLA alleles. We excluded LD associations, including haplotypes and adjacent OLP responses with the same HLA allele association, in which CTL epitopes presumably reside in the overlapping region of these peptides. The most immunodominant OLP, number 54 (NKIVRMYSPVSILDI), was associated with three HLA alleles: A*0207, B*4601 and Cw*0102. "RMYSPVSIL" was previously identified as an A*0207-restricted CTL epitope [26]. All three responsible HLA alleles were found to be in LD. However, the association with B*4601 and Cw*0102 was much stronger than for A*0207 (odds ratio 29.4 in B*4601 and 104 in Cw*0102 vs 5.5 in A*0207) and further analysis including by ^{51}Cr release assay is warranted.

From this study, we have substantially increased information about CTL epitopes in CRF01_AE infection, reporting at least 6 unique CRF01_AE CTL epitope and 7 novel cross-clade epitope candidates. CRF01_AE is a recombinant HIV-1 with Gag derived from subtype A [4], from which CTL epitope information is limited, compared to subtypes B or C. We anticipate that if a more detailed epitope mapping study were to be conducted in subtype A-infected populations, there would be a large number of epitopes cross-recognized between CRF01_AE and subtype A.

Although details of OLP-HLA associations are substantially different between subtypes, interestingly we found a similarity in the immunodominant regions between subtypes. Our data showed that the second half of p24 was the most immunodominant regions, followed by the first half of p17 regions. This finding is consistent with previous reports [13,15,27]. We were concerned that the compatibility between OLP sequences and circulating Gag sequences may vary depending on the conservativeness and influence on the pattern of Gag CTL responses. However, the proportion of gag clones that were completely matched to the amino-acid sequence of OLPs was not associated with the frequency of OLP responses (data not shown).

Cross-clade CTL responses are said to be influenced by the viral sequence variability between subtypes, especially the sequence at anchor positions of the HLA binding motif [4,28–31]. Among the 7 newly identified cross-clade epitope candidates, 6 shared the same sequences with reported epitopes at both the B and F pockets. We also compared sequence compatibility at the anchor positions of the best-defined 12 epitopes, not identified in our study. 11 out of 12 also had compatible sequences at anchor positions, implying that sequence compatibility at anchor positions per se does not predict cross-clade reactivity. Other factors should be considered, such as sequences at flanking regions affecting peptide cleavage by the proteasome [32,33] and epitope-HLA complex recognition by T cell receptors (TCRs) [34,35].

This study has a number of limitations. First, we focused on Gag CTL immune responses and did not investigate whole viral proteins. However, since this type of analysis requires a large number of cells, and the volume of blood that we were able to take was rather limited, we decided to focus on Gag responses, as Gag is known to be the most important viral target. Instead of testing a large number of OLPs individually, we undertook experiments in triplicate, using a matrix system, to improve reliability. However, it would have been ideal if we had obtained enough volume of blood to confirm all responses using the individual peptides. Second, we detected OLP-HLA associations by a statistical method and not by the standard HLA-restriction analysis. This approach is easily influenced by sample size and the impact of LD. Thus our study does not provide direct evidence. Third, we have not yet confirmed these OLP responses with CTL using the ^{51}Cr release assay. However, ELISpot assays are now widely accepted as a technique for mapping CTL epitopes [36]. Fourth, these data are based on single cytokine release of gIFN; we did not evaluate multi-functionality of CTL with other cytokines such as IL2 or TNFα [37].

However, our data indicate the existence of a substantial number of unique CTL epitopes in CRF01_AE infection; it is therefore worth conducting a systematic analysis of CTL epitopes when vaccine trials are undertaken in different populations infected with different subtypes.

Supporting Information

Table S1 HLA allele frequencies in the study population.
(XLS)

Table S2 Gag overlapping peptide responses and their HLA allele associations.
(XLS)

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Author Contributions

Conceived and designed the experiments: BS PS KA. Performed the experiments: NW CB MM. Analyzed the data: MM NT. Contributed reagents/materials/analysis tools: BS PP KA. Wrote the paper: MM KA. Clinical evaluation and patient recruitment: PP. Critical review: TM.

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Impact of the National Access to Antiretroviral Program on the incidence of opportunistic infections in Thailand

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ABSTRACT

The National Access to Antiretroviral Program caused a decline in HIV mortality in Thailand, but its impact on opportunistic infections (OI) remains unknown. The aim of this study was to compare the incidence of different OIs before and after the initiation of highly active antiretroviral therapy (HAART). Data from a prospective cohort at a hospital in northern Thailand were analysed. In total, 704 patients enrolled from July 2000 to October 2002 and not on HAART were followed up until October 2004. In addition, 409 patients who started HAART between April 2002 and January 2004 were followed up for 24 months. The impact of HAART on OIs was analysed using Cox proportional hazard models. HAART was associated with a strong reduction in OIs. The reduction appeared to vary by type: tuberculosis (TB), adjusted hazard ratio (AHR) = 0.2 (95% CI 0.1–0.5); pneumocystis pneumonia (PCP), AHR = 0.03 (95% CI 0.007–0.1); cryptococcal meningitis, AHR = 0.2 (95% CI 0.1–0.5); and penicilliosis, AHR = 0.1 (95% CI 0.06–0.3). In conclusion, HAART was very effective in reducing OIs, especially PCP. TB and cryptococcal meningitis remained frequent in the early phase of antiretroviral drug therapy. More attention to prophylaxis as well as earlier diagnosis and starting treatment for these OIs is recommended.

1. Introduction

Highly active antiretroviral therapy (HAART) has greatly decreased AIDS and AIDS-related mortality in developed countries.[1–3] However, only recently has HAART become more widely available in resource-limited countries. The WHO estimates that more than 4 million people were receiving HAART in middle- and low-income countries at the end of 2008, representing an increase of 36% in 1 year and a 10-fold increase over 5 years.[4] The HIV mortality rate has declined in middle- and low-income countries but is still higher compared with high-income countries, especially in the first few months after starting HAART.[5,6] Thailand has been one of the first Asian countries severely affected by the HIV epidemic since the early 1990 s. The Thai government expanded the antiretroviral drug programme to the national scale in 2004, as the National Access to Antiretroviral Program for People living with HIV/AIDS (NAPHA).[7] This programme rapidly

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increased patient access to HAART by supplying a fixed-dose combination of generic drugs ('GPO-Vir'). As a result, a substantial decline in mortality has been observed (unpublished data).

Since opportunistic infections (OI) are the major cause of death in HIV-infected individuals, the decline in AIDS-related mortality in the HAART era is mainly attributed to the decline in OIs.[8] There are several reports from high-income countries in North America and Europe showing that the introduction of HAART has greatly lowered the incidence of AIDS-defining illnesses.[2,9] It is known that there is a considerable difference in the distribution pattern of OIs in different geographical areas.[10] There are few data on the impact of HAART on OIs from low- and middle-income countries in Asia and Africa. Several studies in Africa investigated the effect of OI prophylaxis on the incidence of OIs.[11,12] However, only two papers evaluated the impact of HAART on OIs. Badri et al.[13] showed that HAART reduced the incidence of HIV-associated tuberculosis (TB) by >80% in a cohort study in South Africa. One study from India reported changes in TB incidence before and after HAART, but they did not quantitatively determine the impact of HAART. Neither of the studies evaluated the incidence of OIs other than TB.[14]

To determine the impact of HAART on AIDS in Thailand, changes in the incidence of different OIs at a government hospital in northern Thailand before and after initiation of the National Access to Antiretroviral Program were examined.

2. Materials and methods

2.1. Study site and study populations

A prospective cohort study was conducted at the HIV Clinic, Day Care Center (DCC) of Lampang Hospital, a government referral hospital with approximately 800 beds situated in the centre of Lampang province in upper northern Thailand. The DCC was established in October 1995 as an outpatient clinic providing treatment, care and support for HIV-infected patients.[15] Recruitment of this cohort started on 6 July 2000 by contacting all HIV patients attending the HIV clinic.[16] Over 95% of patients agreed to participate in the study. All patients were requested to visit the clinic at least every 3 months regardless of the presence of clinical symptoms. If patients developed a clinical event of interest, follow-up was censored at the date of occurrence for this diagnosis, but patients were followed-up for further OIs as long as they survived. In April 2002, the Thai government introduced GPO-Vir (stavudine, lamivudine and nevirapine) into the clinic on a pilot basis and the number of patients receiving GPO-Vir gradually increased. In 2004, the number of patients on HAART rapidly increased as the government integrated the GPO-Vir regimen into the national health insurance service. GPO-Vir became freely available for any HIV patient fulfilling one of the following criteria: low CD4 count of < 200 cells/μl; or diagnosis of AIDS. The incidence of OIs in these patients during the follow-up period was used in the current analysis, with the data from the first part of the cohort (before HAART) serving as a control.

2.2. Data collection

For each participant in the study, sociodemographic data and medical history [HIV-related symptoms, history of antiretroviral therapy (ART), mode of transmission and history of OIs] were obtained at the initial visit by trained research staff through face-to-face interviews using structured questionnaires. In addition, a full blood count, CD4 cell count and viral load were measured. The CD4 cell count was determined using a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA) and HIV viral load was measured using a Cobas Amplicor HIV-1 Monitor Test (Roche Diagnostics, Basel, Switzerland). Diagnosis of OIs was made following the guidelines of Lampang Hospital, which are based on the Thai national guidelines.[17] All clinical information was collected by three physicians specialised in HIV care.

2.3. Clinical management of opportunistic infections

Standard clinical algorithms were used to guide the initiation of prophylactic and therapeutic interventions based on the treatment guidelines of Lampang Hospital (modified from reference[17]). Briefly, as for primary prophylaxis, patients with a CD4 count < 200 cells/mm^3 were given two double-strength tablets of trimethoprim/sulfamethoxazole (TMP/SMX; 80 mg TMP and 400 mg SMX) orally once daily or three times per week for prophylaxis against pneumocystis pneumonia (PCP). The same regimen was administered to prevent toxoplasmosis when the CD4 count was <100 cells/μl. Fluconazole 200 mg orally once daily or 400 mg once a week was given for prophylaxis against cryptococcosis when the CD4 cell count was <100 cells/μl. No primary prophylaxis for TB or *Mycobacterium avium* complex (MAC) infection was given in this study. These treatment guidelines did not change throughout the study.

2.4. Analysis

To analyse the impact of HAART on the incidence of OIs, HIV patients were grouped into before and after receiving HAART. For the 756 patients who were recruited for the cohort between 6 July 2000 and 15 October 2002, information on OIs was collected up to 15 October 2004. For the 409 patients who started GPO-Vir at the clinic between 10 April 2002 and 31 January 2004, information of OIs was collected for 24 months. Incidence rates were calculated by dividing the number of patients developing an event by the number of person-years at risk. To evaluate the impact of HAART on the incidence of OIs, Cox's proportional hazard models with the time since enrolment as time axis was used. Patients who entered the cohort before receiving HAART and who then went on to receive HAART during the follow-up period were included as two separate observations. Therefore, hazard ratios were adjusted using robust standard errors to account for within-person correlation of disease susceptibility. Kaplan–Meier survival plots were used to show the incidence of different OIs in relation to CD4 cell counts at enrolment separately for the before and after HAART groups.

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