Table 1. Cases reported in English of hemophagocytic syndrome after liver transplantation

First author[Ref.]	No.	Age (years)	Sex	Donor type	Diagnosis	Onset (POD)	Causes	Treatment					Prognosis/ outcome
								G-CSF	IVIg	Steroid	Dialysis	Chemotherapy	
Chisuwa[6]	1	9 mo	F	Living	Biliary atresia	15	Unknown	–	+	+	PE	Etoposide	Died
	2	11 mo	M	Living	Biliary atresia	134	EBV	+	–	+	–	–	Died
George[7]	3	10	M	Deceased	Acute liver failure	6 years	EBV	–	–	+	+	Etoposide	Alive
Karasu[8]	4	38	M	Living	HBV/HDV	124	Unknown	+	+	–	PE	–	Alive
Lladó[12]	5	63	M	Deceased	Autoimmune hepatitis	NA	Unknown	–	+	+	–	–	Died
Taniai[9]	6	37	F	Living	Unknown	11	Unknown	–	+	+	PE, CHDF	–	Died
Hardikar[13]	7	26 mo	M	Deceased	Acute liver failure	15	CMV	+	+	–	–	–	Alive
Akamatsu[10]	8	59	M	Living	LC/HCV	138	*Aspergillus*	–	–	–	–	–	Died
	9	49	F	Living	PBC	315	CMV	–	+	+	PE	–	Died
	10	48	F	Living	LC/HCV	50	HCV	+	–	–	–	–	Died
Yoshizumi[11]	11	63	M	Living	PBC	13	Small-for-size syndrome	+	+	+	–	–	Alive
Dharancy[14]	12	49	F	Deceased	(Liver/kidney) polycystic liver/kidney	12	HHV6	–	–	–	–	–	Died
Present cases													
Case 1		57	M	Living	LC/HCV/HCC	32	CMV/HCC	+	+	+	–	–	Died
Case 2		63	M	Living	LC/HBV/HCC	81	Unknown	+	+	+	–	–	Died

mo, months; HBV, hepatitis B virus; HCV, hepatitis C virus; HDV, hepatitis D virus; LC, liver cirrhosis; HCC, hepatocellular carcinoma; POD, postoperative day; G-CSF, granulocyte-colony stimulating factor; IVIg, intravenous immunoglobulin; EBV, Epstein-Barr virus; CMV, cytomegalovirus; PE, plasma exchange; CHDF, continuous hemodiafiltration; NA, not available

the causative agent in our Case 1 was assumed to be CMV, although HCC could also have contributed. Even after removal of the liver, the possibility of far advanced poorly differentiated HCC triggering HPS cannot be excluded.

Hemophagocytic syndrome is defined as a proliferation of phagocytic macrophages in the bone marrow, spleen, or lymph nodes, with clinical findings such as a fever of ≥7 days with peaks ≥38.5°C, cytopenia (at least two of three lineages), and splenomegaly.[1] Therefore, the diagnosis of HPS is based on both clinical and pathological findings. In terms of pathophysiology, in the acute phase, a lymphohistiocytic infiltrate is typically found, most commonly in the spleen, lymph nodes, and bone marrow.[17] Furthermore, the levels of circulating T-cell cytokines and monokines were increased.[18] The view that the inability of natural killer cells and cytotoxic T cells to efficiently terminate an immune response, triggered, for example, by an infectious agent, leads to sustained activation of lymphocytes and macrophages, stems from these observations. The result is widespread hemophagocytosis and the overproduction of cytokines such as interferon-γ, tumor necrosis factor α (TNF-α), interleukin (IL)-1, and IL-6.

Hyperferritinemia (≥1000 ng/ml) and elevated serum LDH (≥1000 IU/l) are important clues to the diagnosis of secondary HPS in febrile cytopenic patients with immunodeficiency.[19,20] The levels of interferon-γ or soluble IL-2 receptor[21] are also thought to be useful predictors of prognosis, while TNF-α or IL-6 has been reported to be related to HPS.[22] However, these markers and other types of cytokines are not specific, especially in the inflammatory environment during the post-transplant course. Furthermore, because the measurement of serum cytokines is time-consuming and expensive, it does not seem practical in LDLT recipients. β2-Microglobulin is a reactive protein derived from activated macrophages,[23] although it may be produced in the process of alloimmunity or excessively filtered into urine as a result of immunosuppressant-induced nephrotoxicity.

Based on a better understanding of the possible role of T lymphocytes and macrophages, combination therapy using cyclosporine, steroids, and etoposide is the established treatment for nontransplant HPS.[2] According to Henzan et al., anticytokine treatment with infliximab for HPS patients not responding to conventional therapy may be a promising option.[24] Although bone marrow or stem cell transplantation with strong chemotherapy has been reported for refractory cases,[2] these treatments have never been reported for HPS after LDLT. The essential treatment for secondary HPS is to manage the underlying disease and hypercytokinemia. Only when the causative infection is controlled can it be possible to achieve remission of IAHS.

G-CSF is used in the treatment of HPS because it affects not only the granulocyte lineage but also other lineages,[25] whereas IVIg has been reported to improve the prognosis of HPS.[26] Based on their successful experience, Karasu et al. suggested a combination therapy of G-CSF and IVIg for treating HPS in LT recipients.[8] Although we adopted the same strategy, both our patients died of multiple organ failure despite the improvement of their pancytopenia.

After solid organ transplantation, high levels of immunosuppression or post-transplant sepsis may trigger the onset of hemophagocytosis. These patients may benefit from an interruption of immunosuppressive therapy, whereas nontransplant HPS patients who are not on immunosuppression therapy may benefit from immunosuppressive treatment. The use of steroid pulse therapy, established for nontransplant HPS patients, should be reconsidered for transplant patients, especially in the early postoperative period when they are receiving stronger immunosuppression.

The previously reported cases describe several treatments (Table 1), despite which only 4 of the 14 patients survived. Thus, it is essential to establish a therapeutic strategy for HPS in LT recipients, modified from the strategy established for nontransplant patients with HPS. Pancytopenia is frequently encountered in LT recipients, caused by viral infection, drugs, and other factors. Although the reasons for pancytopenia are often obscure, bone marrow aspiration should be performed as soon as possible if an LT recipient becomes febrile and is found to have pancytopenia, to rule out HPS because of its poor prognosis. An early diagnosis might improve the outcome of this devastating syndrome. In conclusion, it is important to recognize reactive HPS as a rare but fatal complication that can occur after LT.

References

1. Henter JI, Elinder G, Ost A. Diagnostic guidelines for hemophagocytic lymphohistiocytosis. The FHL Study Group of the Histiocyte Society. Semin Oncol 1991;18:29–33.
2. Imashuku S. Advances in the management of hemophagocytic lymphohistiocytosis. Int J Hematol 2000;72:1–11.
3. Liver Cancer Study Group of Japan. General rules for the clinical and pathological study of primary liver cancer, 2nd English ed. Tokyo: Kanehara; 2003.
4. Egawa H, Teramukai S, Haga H, Tanabe M, Fukushima M, Shimazu M. Present status of ABO-incompatible living donor liver transplantation in Japan. Hepatology 2008;47:143–52.
5. Yoshimura N, Okajima H, Ushigome H, Sakamoto S, Fujiki M, Okamoto M. Current status of organ transplantation in Japan and worldwide. Surg Today 2010;40:514–25.
6. Chisuwa H, Hashikura Y, Nakazawa, Kamijo T, Nakazawa K, Nakayama J, et al. Fatal hemophagocytic syndrome after living-related liver transplantation: a report of two cases. Transplantation 2001;72:1843–6.

7. George TI, Jeng M, Berquist W, Cherry AM, Link MP, et al. Epstein–Barr virus-associated peripheral T-cell lymphoma and hemophagocytic syndrome arising after liver transplantation: case report and review of the literature. Pediatr Blood Cancer 2005;44: 270–6.
8. Karasu Z, Kilic M, Cagirgan S, Lebe E, Yilmaz F, Demirbas T, et al. Hemophagocytic syndrome after living-related liver transplantation. Transplant Proc 2003;35:1482–4.
9. Taniai N, Akimaru K, Kawano Y, Mizuguchi Y, Shimizu T, Takahashi T, et al. Hemophagocytic syndrome after living-donor liver transplantation for fulminant liver failure: a case report. Hepatogastroenterology 2005;52:923–6.
10. Akamatsu N, Sugawara Y, Tamura S, Matsui Y, Hasegawa K, Imamura H, et al. Hemophagocytic syndrome after adult-to-adult living donor liver transplantation. Transplant Proc 2006;38: 1425–8.
11. Yoshizumi T, Taketomi A, Kayashima H, Harada N, Uchiyama H, Yamashita Y, et al. Successful treatment for a patient with hemophagocytic syndrome after a small-for-size graft liver transplantation. Hepatogastroenterology 2008;55:359–62.
12. Lladó L, Figueras J, Comí S, Torras J, Serrano T, Castellote J, et al. Haemophagocytic syndrome after liver transplantation in adults. Transpl Int 2004;17:221–3.
13. Hardikar W, Pang K, Al-Hebbi H, Curtis N, Couper R. Successful treatment of cytomegalovirus-associated haemophagocytic syndrome following paediatric orthotopic liver transplantation. J Paediatr Child Health 2006;42:389–91.
14. Dharancy S, Crombe V, Copin MC, Boleslawski E, Bocket L, Declerck N, et al. Fatal hemophagocytic syndrome related to human herpesvirus-6 reinfection following liver transplantation: a case report. Transplant Proc 2008;40:3791–3.
15. Risdall RJ, McKenna RW, Nesbit ME, Krivit W, Balfour HH Jr, Simmons RL, et al. Virus-associated hemophagocytic syndrome: a benign histiocytic proliferation distinct from malignant histiocytosis. Cancer 1979;44:993–1002.
16. Sakai T, Shiraki K, Deguchi M, Itoh N, Konishi T, Takase K, et al. Hepatocellular carcinoma associated with hemophagocytic syndrome. Hepatogastroenterology 2001;48:1464–6.
17. Hansmann ML, Rontogianni D, Jankaschaub GE, Wacker HH, Hinkeldey K, Radzun HJ. Familial hemophagocytic lymphohistiocytosis macrophages showing immunohistochemical properties of activated macrophages and T-accessory cells. Pediatr Hematol Oncol 1989;6:237–46.
18. Henter JI, Elinder G, Soder O, Hansson M Andersson B, Andersson U. Hypercytokinemia in familial hemophagocytic lymphohistiocytosis. Blood 1991;78:2918–22.
19. Imashuku S. Differential diagnosis of hemophagocytic syndrome: underlying disorders and selection of the most effective treatment. Int J Hematol 1997;66:135–51.
20. Koduri PR, Carandang G, DeMarais P, Patel AR. Hyperferritinemia in reactive hemophagocytic syndrome report of four adult cases. Am J Hematol 1995;49:247–9.
21. Imashuku S, Hibi S, Sako M, Ishida Y, Mugishima H, Chen J, et al. Soluble interleukin-2 receptor: a useful prognostic factor for patients with hemophagocytic lymphohistiocytosis. Blood 1995; 86;4706–7.
22. Billiau AD, Roskams T, Van Damme-Lombaerts R, Matthys P, Wouters C. Macrophage activation syndrome: characteristic findings on liver biopsy illustrating the key role of activated, IFN-gamma-producing lymphocytes and IL-6- and TNF-alpha-producing macrophages. Blood 2005;105:1648–51.
23. Hibi S, Ikushima S, Fujiwara F, Hashida T, Tsunamoto K, Todo S, et al. Serum and urine beta-2-microglobulin in hemophagocytic syndrome. Cancer 1995;75:1700–5.
24. Henzan T, Nagafuji K, Tsukamoto H, Miyamoto T, Gondo H, Imashuku S, et al. Success with infliximab in treating refractory hemophagocytic lymphohistiocytosis. Am J Hematol 2006;81:59–61.
25. Azuma E, Tabata N, Shibata T, Komada Y, Ito M, Atsumi S, et al. G-CSF enhanced granulocyte production in a child with severe neutropenia in the setting of fatal virus-associated hemophagocytic syndrome. Am J Hematol 1990;35:266–8.
26. Gill DS, Spencer A, Cobcroft RG. High-dose gamma-globulin therapy in the reactive haemophagocytic syndrome. Br J Haematol 1994;88:204–6.

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PLoS one

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

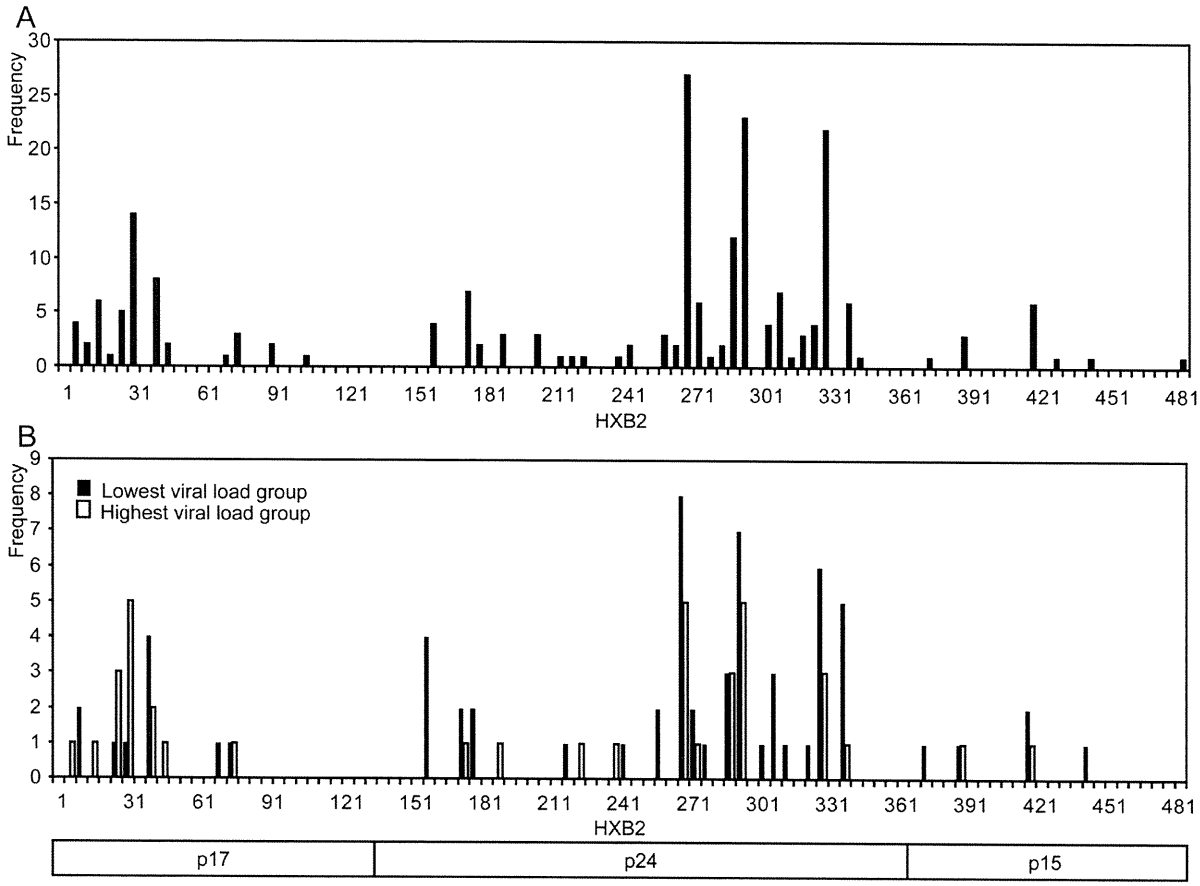

Figure 1. Pattern of CRF01_AE Gag CTL responses. Frequencies of overlapping peptide (OLP) responses in 112 individuals are shown (A); Frequencies of OLP responses in the lowest viral load group (lowest quartile, n = 34) and the highest viral load group (highest quartile, n = 34) were compared (B).
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243–1,057) 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

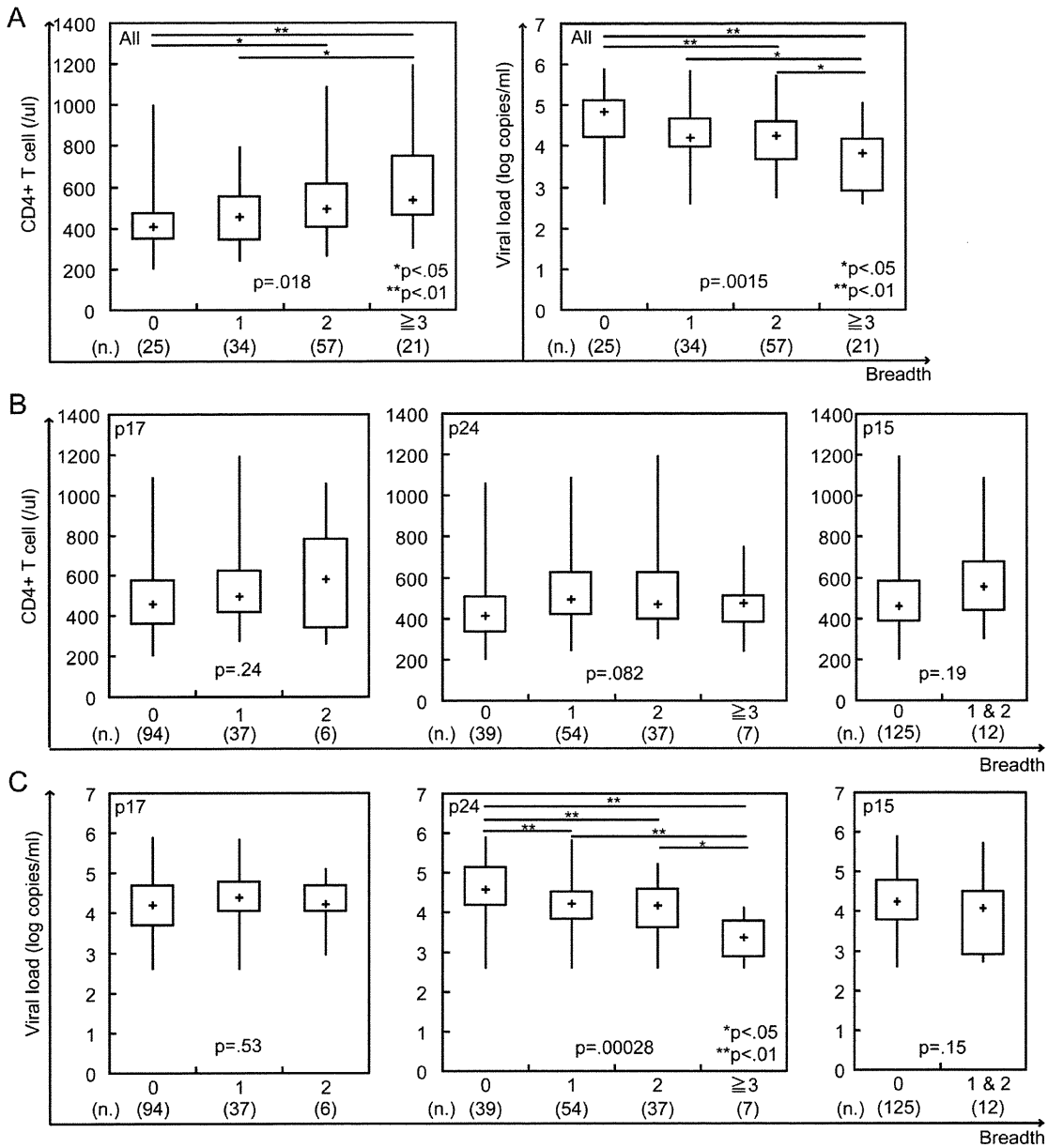

Figure 2. ELISpot breadth is associated with CD4+ T cell count and viral load. The associations between ELISpot breadth (the number of reacting OLP) and CD4+ T cell count or viral load were analyzed using the Kruskal-Wallis test (A). The p17, p24 or p15 site-specific ELISpot breadth was also compared 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

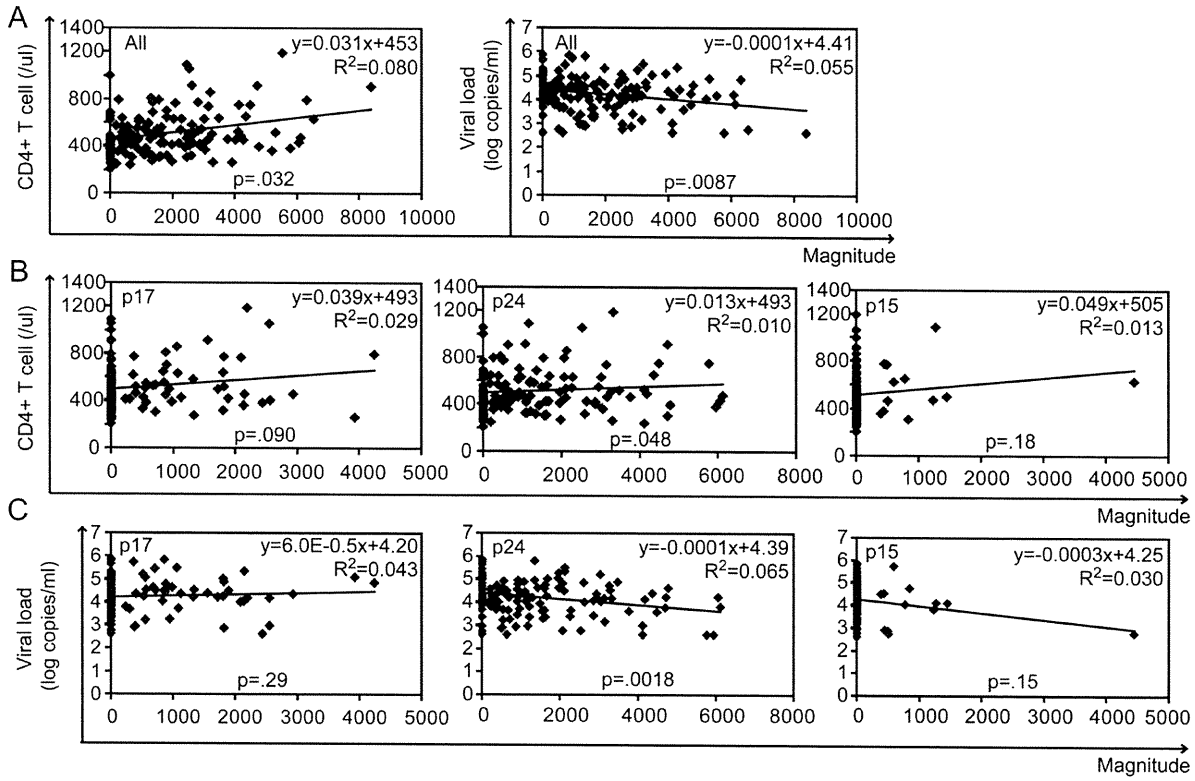

Figure 3. ELISpot magnitude is associated with CD4+ T cell count and viral load. The associations between ELISpot magnitude (total SFU per 1.0 M PBMC) and CD4+ T cell count or viral load were analyzed by Spearman's correlation (A). The p17, p24 or p15 site-specific response was also compared with CD4+ 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

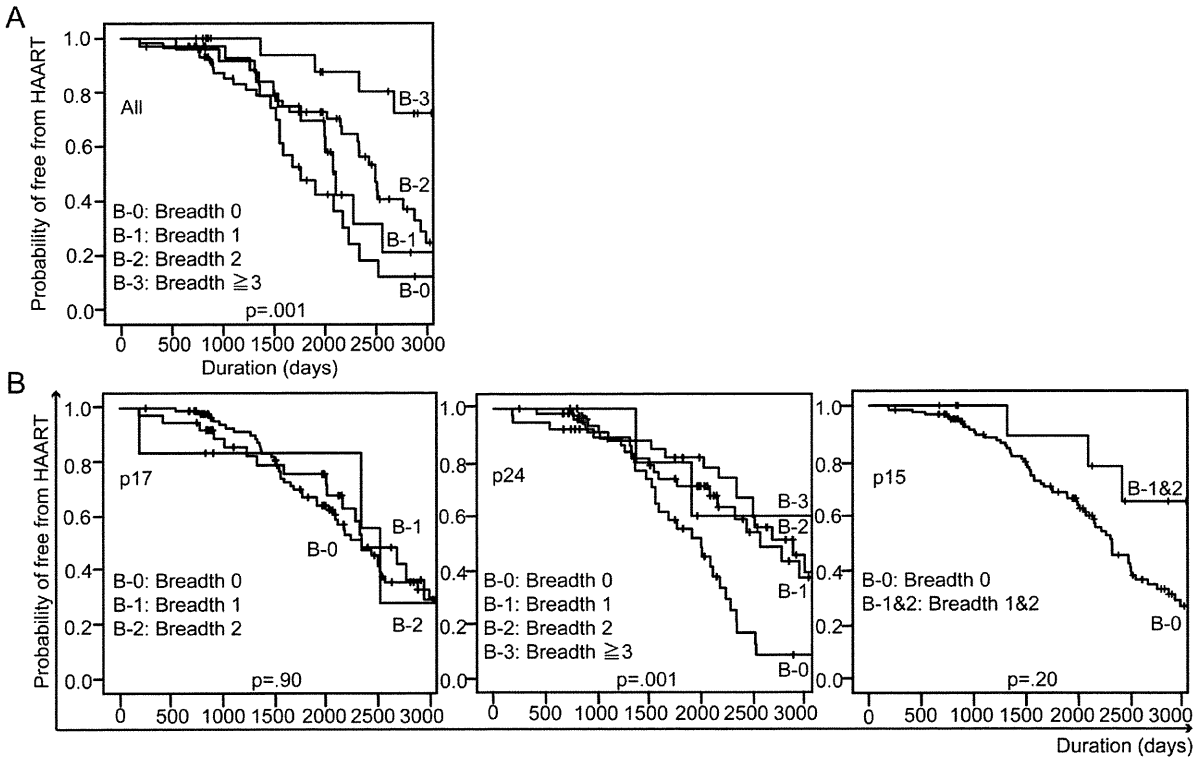

Figure 4. ELISpot breadth is related to delayed initiation of antiretroviral therapy. The impact of ELISpot breadth on antiretroviral therapy initiation was evaluated by Kaplan-Meier analysis, using the log rank test (A). The effect of p17, p24 or p15 site-specific ELISpot breadth was also analyzed (B).
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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.

References

1. McMichael AJ, Borrow P, Tomaras GD, Goonetilleke N, Haynes BF (2010) The immune response during acute HIV-1 infection: clues for vaccine development. Nat Rev Immunol 0: 11–23.
2. Kaslow RA, Carrington M, Apple R, Park L, Muñoz A, et al. (1996) Influence of combinations of human major histocompatibility complex genes on the course of HIV-1 infection. Nat Med 2: 405–11.
3. Kiepiela P, Leslie AJ, Honeyborne I, Ramduth D, Thobakgale C, et al. (2004) Dominant influence of HLA-B in mediating the potential co-evolution of HIV and HLA. Nature 432: 769–75.
4. Buonaguro L, Tornesello ML, Buonaguro FM (2007) Human immunodeficiency virus type 1 subtype distribution in the worldwide epidemic: pathogenetic and therapeutic implications. J Virol 81: 10209–19.
5. Dorrell L, Willcox BE, Jones EY, Gillespie G, Njai H, et al. (2001) Cytotoxic T lymphocytes recognize structurally diverse, clade-specific and cross-reactive peptides in human immunodeficiency virus type-1 gag through HLA-B53. Eur J Immunol 31: 1747–56.
6. Ferrari G, Humphrey W, McElrath MJ, Excler JL, Duliege AM, et al. (1997) Clade B-based HIV-1 vaccines elicit cross-clade cytotoxic T lymphocyte reactivities in uninfected volunteers. Proc Natl Acad Sci USA 94: 1396–401.
7. Kiepiela P, Ngumbela K, Thobakgale C, Ramduth D, Honeyborne I, et al. (2007) CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. Nat Med 13: 46–53.
8. Rerks-Ngarm S, Pitisuttithum P, Nitayaphan S, Kaewkungwal J, Chiu J, et al. (2009) Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. N Engl J Med 361: 2209–20.
9. Addo MM, Yu XG, Rathod A, Cohen D, Eldridge RL, et al. (2003) Comprehensive epitope analysis of human immunodeficiency virus type 1 (HIV-1)-specific T-cell responses directed against the entire expressed HIV-1 genome demonstrate broadly directed responses, but no correlation to viral load. J Virol 77: 2081–92.
10. Kern F, Surel IP, Faulhaber N, Frömmel C, Schneider-Mergener J, et al. (1999) Target structures of the CD8(+)-T-cell response to human cytomegalovirus: the 72-kilodalton major immediate-early protein revisited. J Virol 73: 8179–84.
11. Masemola A, Mashishi T, Khoury G, Mohube P, Mokgotho P, et al. (2004) Hierarchical targeting of subtype C human immunodeficiency virus type 1 proteins by CD8+ T cells: correlation with viral load. J Virol 78: 3233–43.
12. Brumme Z, Wang B, Nair K, Brumme C, de Pierres C, et al. (2009) Impact of select immunologic and virologic biomarkers on CD4 cell count decrease in patients with chronic HIV-1 subtype C infection: results from Sinikithemba Cohort, Durban, South Africa. Clin Infect Dis 49: 956–64.
13. Geldmacher C, Currier JR, Herrmann E, Haule A, Kuta E, et al. (2007) CD8 T-cell recognition of multiple epitopes within specific Gag regions is associated with maintenance of a low steady-state viremia in human immunodeficiency virus type 1-seropositive patients. J Virol 81: 2440–8.
14. Novitsky V, Gilbert P, Peter T, McLane MF, Gaolekwe S, et al. (2003) Association between virus-specific T-cell responses and plasma viral load in human immunodeficiency virus type 1 subtype C infection. J Virol 77: 882–90.
15. Zuñiga R, Lucchetti A, Galvan P, Sanchez S, Sanchez C, et al. (2006) Relative dominance of Gag p24-specific cytotoxic T lymphocytes is associated with human immunodeficiency virus control. J Virol 80: 3122–5.
16. Gesprasert G, Wichukchinda N, Mori M, Shiino T, Auwanit W, et al. (2010) HLA-associated immune pressure on Gag protein in CRF01_AE-infected individuals and its association with plasma viral load. PLoS One 5: e11179.
17. Martinez-Picado J, Prado JG, Fry EE, Pfafferott K, Leslie A, et al. (2006) Fitness cost of escape mutations in p24 Gag in association with control of human immunodeficiency virus type 1. J Virol 80: 3617–23.
18. Sacha JB, Chung C, Rakasz EG, Spencer SP, Jonas AK, et al. (2007) Gag-specific CD8+ T lymphocytes recognize infected cells before AIDS-virus integration and viral protein expression. J Immunol 178: 2746–54.
19. Goulder PJ, Watkins DI (2008) Impact of MHC class I diversity on immune control of immunodeficiency virus replication. Nat Rev Immunol 8: 619–30.
20. Yusim K, Kesmir C, Gaschen B, Addo MM, Altfeld M, et al. (2002) Clustering patterns of cytotoxic T-lymphocyte epitopes in human immunodeficiency virus type 1 (HIV-1) proteins reveal imprints of immune evasion on HIV-1 global variation. J Virol 76: 8757–68.
21. Briggs JA, Simon MN, Gross I, Kräusslich HG, Fuller SD, et al. (2004) The stoichiometry of Gag protein in HIV-1. Nat Struct Mol Biol 11: 672–5.
22. Julg B, Williams KL, Reddy S, Bishop K, Qi Y, et al. (2010) Enhanced anti-HIV functional activity associated with Gag-specific CD8 T-cell responses. J Virol 84: 5540–9.
23. Kaslow RA, Dorak T, Tang JJ (2005) Influence of Host Genetic Variation on Susceptibility to HIV Type 1 Infection. J Infect Dis 191: S68–77.
24. Lieberman J, Fabry JA, Fong DM, Parkerson GR, 3rd (1997) Recognition of a small number of diverse epitopes dominates the cytotoxic T lymphocytes response to HIV type 1 in an infected individual. AIDS Res Hum Retroviruses 13: 383–92.
25. Miura T, Brockman MA, Schneidewind A, Lobritz M, Pereyra F, et al. (2009) HLA-B57/B*5801 human immunodeficiency virus type 1 elite controllers select for rare gag variants associated with reduced viral replication capacity and strong cytotoxic T-lymphocyte [corrected] recognition. J Virol 83: 2743–55.
26. Seth A, Yasutomi Y, Jacoby H, Callery JC, Kaminsky SM, et al. (2000) Evaluation of a lipopeptide immunogen as a therapeutic in HIV type 1-seropositive individuals. AIDS Res Hum Retroviruses 16: 337–43.
27. Thakar MR, Bhonge LS, Lakhashe SK, Shankarkumar U, Sane SS, et al. (2005) Cytolytic T lymphocytes (CTLs) from HIV-1 subtype C-infected Indian patients recognize CTL epitopes from a conserved immunodominant region of HIV-1 Gag and Nef. J Infect Dis 192: 749–59.
28. Geels MJ, Dubey SA, Anderson K, Baan E, Bakker M, et al. (2005) Broad cross-clade T-cell responses to gag in individuals infected with human immunodeficiency virus type 1 non-B clades (A to G): importance of HLA anchor residue conservation. J Virol 79: 11247–58.
29. Gudmundsdotter L, Bernasconi D, Hejdeman B, Sandstrom E, Alaeus A, et al. (2008) Cross-clade immune responses to Gag p24 in patients infected with different HIV-1 subtypes and correlation with HLA class I and II alleles. Vaccine 26: 5182–7.

30. Malhotra U, Nolin J, Mullins JI, McElrath MJ (2007) Comprehensive epitope analysis of cross-clade Gag-specific T-cell responses in individuals with early HIV-1 infection in the US epidemic. Vaccine 25: 381–90.
31. Yu XG, Lichterfeld M, Perkins B, Kalife E, Mui S, et al. (2005) High degree of inter-clade cross-reactivity of HIV-1-specific T cell responses at the single peptide level. AIDS 19: 1449–56.
32. Yokomaku Y, Miura H, Tomiyama H, Kawana-Tachikawa A, Takiguchi M, et al. (2004) Impaired processing and presentation of cytotoxic-T-lymphocyte (CTL) epitopes are major escape mechanisms from CTL immune pressure in human immunodeficiency virus type 1 infection. J Virol 78: 1324–32.
33. Tenzer S, Wee E, Burgevin A, Stewart-Jones G, Friis L (2009) Antigen processing influences HIV-specific cytotoxic T lymphocyte immunodominance. Nat Immunol 10: 636–46.
34. Dong T, Stewart-Jones G, Chen N, Easterbrook P, Xu X, et al. (2004) HIV-specific cytotoxic T cells from long-term survivors select a unique T cell receptor. J Exp Med 200: 1547–57.
35. Meyer-Olson D, Brady KW, Bartman MT, O'Sullivan KM, Simons BC, et al. (2006) Fluctuations of functionally distinct CD8+ T-cell clonotypes demonstrate flexibility of the HIV-specific TCR repertoire. Blood 107: 2373–83.
36. Streeck H, Frahm N, Walker BD (2009) The role of IFN-gamma Elispot assay in HIV vaccine research. Nat Protoc 4: 461–9.
37. Betts MR, Nason MC, West SM, De Rosa SC, Migueles SA, et al. (2006) HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. Blood 107: 4781–9.

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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 1990s. 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 increased patient access to HAART by supplying a fixed-dose

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combination of generic drugs ('GPO-Vir'). As a result, a substantial decline in mortality has been observed (P. Pathipvanich et al., 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 the Thai national guidelines[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 proportional hazard models with the time since enrolment as time axis were 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.

Table 1
Baseline characteristics of patients in the before HAART and after HAART groups

Characteristic	Before HAART group (n = 639)	After HAART group (n = 409) [a]
Age [median (IQR)]	32 (29–37)	33 (30–38)
Male gender [n (%)]	267 (41.8)	184 (45.0)
Clinical status [n (%)]		
Asymptomatic	334 (52.3)*	144 (35.2)*
Non-AIDS symptomatic	115 (18.0)	79 (19.3)
AIDS symptomatic	190 (29.7)*	186 (45.5)*
Previous ART [n (%)] [b]	148 (23.2)	101 (24.7)
Baseline CD4 cell count (cells/μl) [median (IQR)]	152 (25–348)*	44 (15–110)*
Baseline viral load (copies/ml) [median (IQR)] [c]	153 448 (33 058–5 189 295)	187 577 (62 791–490 282)
Previous OIs [n (%)]		
Tuberculosis [d]	71 (11.1)	53 (13.0)
Pneumocystis pneumonia	60 (9.4)	61 (14.9)*
Cryptococcal meningitis	51 (8.0)	49 (12.0)
Penicilliosis	19 (3.0)	35 (8.6)
Oesophageal candidiasis	8 (1.3)	47 (11.5)*
Herpes zoster	80 (12.5)	51 (12.5)
Toxoplasma encephalitis	18 (2.8)	8 (2.0)
Cytomegalovirus retinitis	12 (1.9)	25 (6.1)*

HAART: highly active antiretroviral therapy; IQR: interquartile range; ART: antiretroviral therapy; OIs: opportunistic infections.
[a] After HAART group includes new patients as well as those who were enrolled in the before HAART group and who then started HAART.
[b] Experience with ART is limited to mono or dual therapy.
[c] Viral load data were available for only 274 patients in the after HAART group.
[d] Tuberculosis includes both pulmonary and extrapulmonary tuberculosis.
* Statistically significant (P < 0.05).

Results were presented as hazard ratios with 95% CIs. Statistical analyses were conducted using STATA version 10.0 (StataCorp LP, College Station, TX, USA).

3. Results

3.1. Patient characteristics

Of 756 HIV-infected persons recruited before GPO-Vir was introduced, 36 self-funded patients who were receiving HAART before recruitment were excluded. In addition, 16 patients who visited only once at enrolment and who died shortly after were also excluded from the analysis. Follow-up data on OIs were available for 639 (90.8%) of the remaining 704 patients. Total follow-up time was 1024.5 person-years of observation (PYO), with a median follow up of 476 days [interquartile range (IQR) 195–917 days]. During the observation period, 263 patients (41.2%) died, resulting in a mortality rate of 25.7/100 PYO (95% CI 22.6–28.8/100 PYO) in this group. In patients receiving HAART, the total duration of follow-up was 696.7 PYO from the time of treatment initiation, with a median follow-up duration of 720 days (IQR 677–722 days). During the observation period 32 patients died, resulting in a mortality rate of 4.6/100 PYO (95% CI 3.00–6.58/100 PYO), as described previously.[18]

The assumed transmission route was heterosexual in the majority of study patients (95%), with no change over time. Table 1 summarises the baseline characteristics of patients not receiving and receiving HAART. Demographic characteristics such as age and sex were similar. The proportion of patients who had previously received mono or dual ART was also similar between the groups. Patients in the HAART group were more likely to have AIDS or HIV-related symptoms and had a much lower CD4 cell count at enrolment or treatment initiation. Previous OIs (before enrolment) tended to be more common in patients receiving HAART.

3.2. Incidence rate of opportunistic infections and impact of HAART

Table 2 shows incidence rates and hazard ratios of different OIs according to HAART treatment status. In the before HAART group, TB was the most common OI, followed by PCP, cryptococcal meningitis and penicilliosis. In the HAART group, TB and cryptococcal meningitis were the two most common OIs, followed by penicilliosis. PCP was rare in patients on HAART. The incidence of cytomegalovirus (CMV) retinitis remained approximately stable after the introduction of HAART, but numbers were low. In univariate Cox regression analysis, all OIs combined decreased by 60%.

Multivariate Cox regression analysis revealed a great benefit of HAART (Table 2). After adjustment for baseline CD4 cell count, HAART reduced the incidence rate of all OIs by 80%. Further adjustment for age, gender, previous ART and AIDS-related symptoms in the full model had little additional effect on the hazard ratios (Table 2). The reduction in the incidence rate appeared to vary between OIs. The reduction in PCP incidence with HAART was the most substantial, with the reduction in TB and cryptococcal meningitis being significantly lower (P < 0.05). Exclusion of those patients from the HAART group who were enrolled before HAART was available and then went on to receive HAART (n = 195) did not result in marked changes in the hazard ratios.

Approximately 50% of cases of cryptococcal meningitis (8/15; 53.3%) and CMV retinitis (5/11; 45.5%) occurred within the first 2 months after the initiation of HAART with GPO-Vir. Approximately 90% of TB and herpes zoster cases occurred within 1 year, with a median of 175 days (range

Table 2
Incidence of opportunistic infections among HIV-infected patients in the before HAART and after HAART groups

Opportunistic infection	Before HAART group (n = 639)		After HAART group (n = 409)		HR (95% CI) [a]	AHR (95% CI) [b]
	Frequency	Incidence rate/100 PYO (95% CI)	Frequency	Incidence rate/100 PYO (95% CI)		
Tuberculosis [c]	59	5.9 (4.4–7.4)	17	2.5 (1.3–3.7)	0.4 (0.2–0.8)	0.2 (0.1–0.5)
Pneumocystis pneumonia	47	4.7 (3.3–6.0)	2	0.3 (–0.1 to 0.7)	0.06 (0.01–0.2)	0.03 (0.007–0.1)
Cryptococcal meningitis	41	4.2 (2.9–5.5)	15	2.2 (1.1–3.3)	0.5 (0.3–0.9)	0.2 (0.1–0.5)
Penicilliosis	35	3.5 (2.3–4.6)	9	1.3 (0.5–2.2)	0.4 (0.2–0.8)	0.1 (0.06–0.3)
Oesophageal candidiasis	19	1.9 (1.0–2.7)	3	0.4 (–0.1 to 0.9)	0.2 (0.06–0.8)	0.1 (0.02–0.5)
Herpes zoster	40	4.0 (2.7–5.2)	13	1.9 (0.9–3.0)	0.6 (0.3–1.1)	0.5 (0.2–1.0)
Toxoplasma encephalitis	12	1.2 (0.5–1.9)	7	1.0 (0.3–1.8)	0.8 (0.3–2.1)	0.4 (0.1–1.5)
Cytomegalovirus retinitis	18	1.8 (1.0–2.6)	11	1.6 (0.7–2.6)	1.0 (0.4–2.4)	0.6 (0.2–1.7)
All AIDS-defining illnesses	180	19.1 (16.3–21.9)	53	8.2 (6.0–10.4)	0.4 (0.3–0.6)	0.2 (0.1–0.3)

HAART: highly active antiretroviral therapy; PYO: person-years of observation; HR: hazard ratio; AHR: adjusted hazard ratio.
[a] Used robust standard errors.
[b] Adjusted by baseline CD4 cell count, gender, age, AIDS-related symptoms and antiretroviral therapy history.
[c] Includes both pulmonary and extrapulmonary tuberculosis.

51–532 days; IQR 100–274 days) for TB and 125 days (range 9–615 days; IQR 109–195 days) for herpes zoster. In the 189 patients who developed OIs before HAART was available 49 patients (25.9%) experienced more than one OI, whereas in the 53 patients who developed OIs in the HAART group only 6 (11.3%) were diagnosed with multiple OIs.

3.3. *Opportunistic infection-free survival curves*

Figure 1 shows Kaplan–Meier OI-free survival curves for the four most common OIs (TB, PCP, cryptococcal meningitis and penicilliosis) for patients not receiving and receiving HAART, stratified by baseline CD4 cell count. Among the patients in the before HAART group, as expected a lower CD4 cell count at baseline was strongly associated with the development of OIs, except for TB for which the disease-free survival curves overlapped in the lowest and middle CD4 strata. All OIs were rare in the higher CD4 stratum.

In the highest CD4 stratum of patients on HAART, none of the patients developed any of the four OIs. CD4 cell count was associated with TB and cryptococcal meningitis. PCP and penicilliosis were rare in all CD4 count strata.

4. Discussion

This study demonstrates a substantial reduction in the incidence of major OIs following the introduction of HAART at a government referral hospital in northern Thailand.

Compared with reports from high-income countries, it was found that the incidence of OIs was higher both before and after starting HAART. The Swiss HIV Cohort Study reported an overall incidence of AIDS-related OIs within 6 months before and within 15 months of starting HAART of 15.1 and 3.6 incidence per 100 PYO, respectively.[19] The incidence of OIs even after introduction of HAART was twice as high in Thailand than in Europe. We believe that one of the reasons for the high incidence of OIs in this cohort was the low baseline CD4 cell count, as this is one of the strongest risk factor for OIs according to our results and studies elsewhere.[20,21] Another explanation might be the high incidences of TB, cryptococcal meningitis and penicilliosis. Whilst none of these OIs are common in high-income countries, TB is the most common OI and also the leading cause of mortality in HIV-infected patients in resource-limited settings.[22,23] In a cohort study focused on TB in South Africa, the overall incidence of TB among HIV-infected individuals on HAART was 2.4/100 PYO, similar to the current cohort,[13] although the TB incidence in the present cohort might have been particularly high since the median CD4 cell count was lower than in the South Africa cohort.

In resource-limited settings, most patients often present late to ART programmes, with low median CD4 cell counts, a high risk of new HIV-related diseases and high early mortality. During the first year of study, between 8% and 26% of patients have been shown to die during the first year of HAART, with most deaths occurring during the first few months.[6] TB and cryptococcal meningitis are leading causes of early mortality, accounting for up to 20% of all deaths[24] in high HIV prevalence regions.

Previous studies on the incidence of penicilliosis or cryptococcal meningitis in developing countries are scarce.[25,26] The high incidence of fungal OIs such as cryptococcal meningitis and penicilliosis in the present cohort appears to be typical for northern Thailand, southern China and northern Vietnam.[21] In contrast, MAC infection and Kaposi sarcoma, which were reported to be relatively common in high-income countries, were not common in this study.[19] Kaposi sarcoma is known to be rare in Thailand;[27] in fact, no case of Kaposi sarcoma has been diagnosed since the DCC of Lampang Hospital was established in 1995. The prevalence of human herpes virus type 8 (HHV8) may be lower among the heterosexual population in northern Thailand. MAC infection may be underdiagnosed due to the difficulty of confirming the pathogen in blood culture.

Similar to previous reports from the USA, Canada and Europe,[1,19,28] there was a major decline in the incidence of almost every OI following initiation of HAART in the present study. We are unaware of studies from middle- or low-income countries evaluating the impact of HAART on individual OIs other than TB with which our results can be compared.[14,29] In the present study, reduction in the incidence of TB was consistent with a report from South Africa.[13] Some evidence that the effect of HAART on PCP was stronger than the effect on TB was also found; TB and cryptococcal meningitis remained quite common after the

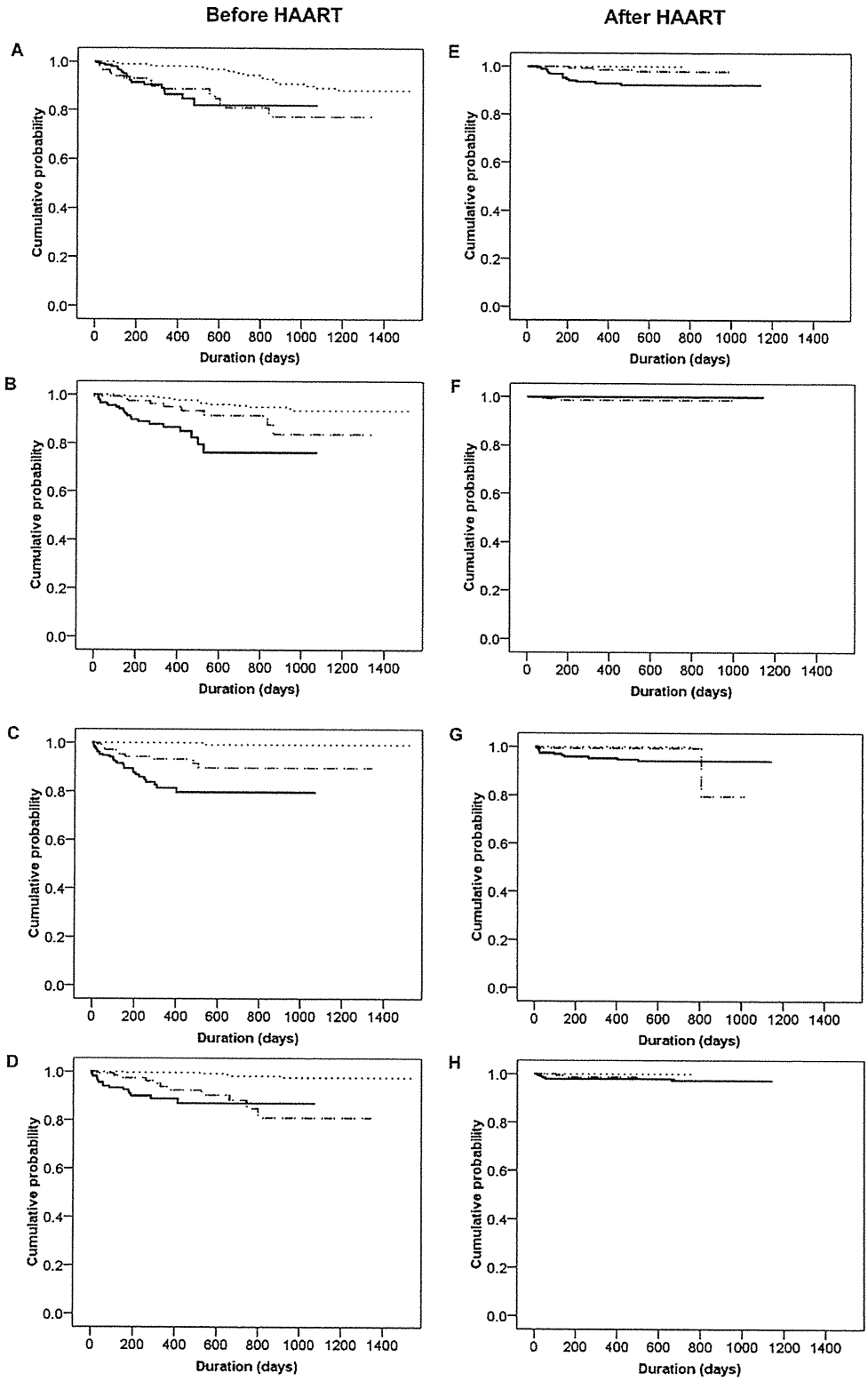

Figure 1. Kaplan–Maier estimates of patients diagnosed with major opportunistic infections before and after highly active antiretroviral drug therapy (HAART) stratified by baseline CD4 cell count: solid thick line, CD4 cell count <50 cells/μl; dashed line, 50–199 cells/μl; and dotted line, ≥200 cells/μl. Cumulative probability indicates patients free from tuberculosis (A and E), pneumocystis pneumonia (B and F), cryptococcal meningitis (C and G) and penicilliosis (D and H).

initiation of HAART, although the majority of such cases occurred within 1 year after starting HAART.

In contrast to TB and cryptococcal meningitis, PCP almost disappeared. Primary prophylaxis might have been more effective against PCP compared with prophylaxis against cryptococcal meningitis and toxoplasmosis. However, the same practice was applied for patients before receiving HAART. The Swiss HIV Cohort Study also showed that the decline in incidence was most pronounced for Kaposi sarcoma, followed by PCP.[19] In the current study, only marginal change in the incidence of CMV retinitis before and after HAART was noted, possibly because CMV retinitis before HAART tended to be underdiagnosed. Because of the limited availability of ophthalmologists in most government HIV clinics, ophthalmological screening is not routine practice. One-half of CMV cases were diagnosed shortly after starting HAART as they developed visual symptoms, which might be due to the immune reconstitution inflammatory syndrome (IRIS).[30] CMV infection is known to be one of the most common OIs associated with IRIS.[31] The relatively high incidence of CMV retinitis in the current cohort, recognised shortly after the initiation of HAART, might therefore be linked to IRIS.

This study is limited by the before/after design without a concurrent control group as in a randomised controlled trial. Patients enrolled after the introduction of HAART tended to have more unfavourable clinical characteristics, which may contribute to understating the effect of HAART. The effect size was therefore adjusted for the CD4 cell count as the most important predictor of OI. Additional adjustment for other potential confounders had little effect on the hazard ratios, but some residual confounding may still be present.

In the present study, the before and after HAART groups were followed-up at the same clinic, and throughout the observation period only three clinicians were involved in the management of patients. Knowledge of treatment allocation by clinicians assessing clinical symptoms may lead to bias towards a greater impact of HAART because of doctors' expectations as to its effectiveness. On the other hand, improved treatment options for a previously fatal disease can raise the motivation of staff to diagnose OIs more accurately, biasing the effect of HAART toward null. To minimise the risk of observer bias, the clinicians followed a local standardised guideline developed to suit the management of HIV patients in Lampang Hospital.

In summary, a substantial reduction in incidence of individual OIs was seen after starting ART in this setting. PCP almost disappeared among patients on GPO-Vir, whereas TB and cryptococcal meningitis remained relatively common OIs especially within the first year of starting HAART. In light of these findings, chemoprophylaxis, screening, and early diagnosis and treatment for TB and cryptococcal meningitis deserve attention in the HAART era among HIV patients with low CD4 cell counts in resource-limited settings, especially in northern Thailand.

Authors' contributions: AR and NT contributed equally to this work. AR, PP, NT, WA, PS and KA conceived the study and designed the study protocol; AR, WP and PP carried out the clinical assessment and management; PS and WA helped with organisation and execution of the study; NT contributed to data collection; AR, NT and KA analysed the data; all authors contributed to interpretation of the data; AR, NT and KA drafted the manuscript; W-PS and SH provided statistical support. All authors read and revised the manuscript critically for intellectual content and approved the final version. KA is guarantor of the paper.

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References

1. Palella Jr FJ, Delaney KM, Moorman AC, Loveless MO, Fuhrer J, Satten GA, et al. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators. *N Engl J Med* 1998;**338**:853–60.
2. Mocroft A, Vella S, Benfield TL, Chiesi A, Miller V, Gargalianos P, et al. Changing patterns of mortality across Europe in patients infected with HIV-1. EuroSIDA Study Group. *Lancet* 1998;**352**:1725–30.
3. Egger M, Hirschel B, Francioli P, Sudre P, Wirz M, Flepp M, et al. Impact of new antiretroviral combination therapies in HIV infected patients in Switzerland: prospective multicentre study. Swiss HIV Cohort Study. *BMJ* 1997;**315**:1194–9.
4. WHO. *Towards universal access: scaling up priority HIV/AIDS interventions in the health sector. Progress report June 2008*. Geneva: World Health Organization; 2008.
5. Braitstein P, Brinkhof MW, Dabis F, Schechter M, Boulle A, Miotti P, et al. Mortality of HIV-1-infected patients in the first year of antiretroviral therapy: comparison between low-income and high-income countries. *Lancet* 2006;**367**:817–24.
6. Lawn SD, Harries AD, Anglaret X, Myer L, Wood R. Early mortality among adults accessing antiretroviral treatment programmes in sub-Saharan Africa. *AIDS* 2008;**22**:1897–908.
7. Chasombat S, McConnell MS, Siangphoe U, Yuktanont P, Jirawattanapisal T, Fox K, et al. National expansion of antiretroviral treatment in Thailand, 2000-2007: program scale-up and patient outcomes. *J Acquir Immune Defic Syndr* 2009;**50**:506–12.
8. Smit C, Geskus R, Walker S, Sabin C, Coutinho R, Porter K, et al. Effective therapy has altered the spectrum of cause-specific mortality following HIV seroconversion. *AIDS* 2006;**20**:741–9.
9. Kaplan JE, Hanson D, Dworkin MS, Frederick T, Bertolli J, Lindegren ML, et al. Epidemiology of human immunodeficiency virus-associated opportunistic infections in the United States in the era of highly active antiretroviral therapy. *Clin Infect Dis* 2000;**30**(Suppl 1):S5–14.
10. Yazdanpanah Y, Chene G, Losina E, Goldie SJ, Merchadou LD, Alfandari S, et al. Incidence of primary opportunistic infections in two human immunodeficiency virus-infected French clinical cohorts. *Int J Epidemiol* 2001;**30**:864–71.

11. Holmes CB, Losina E, Walensky RP, Yazdanpanah Y, Freedberg KA. Review of human immunodeficiency virus type 1-related opportunistic infections in sub-Saharan Africa. *Clin Infect Dis* 2003;**36**:652–62.
12. Holmes CB, Wood R, Badri M, Zilber S, Wang B, Maartens G, et al. CD4 decline and incidence of opportunistic infections in Cape Town, South Africa: implications for prophylaxis and treatment. *J Acquir Immune Defic Syndr* 2006;**42**:464–9.
13. Badri M, Wilson D, Wood R. Effect of highly active antiretroviral therapy on incidence of tuberculosis in South Africa: a cohort study. *Lancet* 2002;**359**:2059–64.
14. Kumarasamy N, Solomon S, Chaguturu SK, Cecelia AJ, Vallabhaneni S, Flanigan TP, et al. The changing natural history of HIV disease: before and after the introduction of generic antiretroviral therapy in southern India. *Clin Infect Dis* 2005;**41**:1525–8.
15. Pathipvanich P, Ariyoshi K, Rojanawiwat A, Wongchoosie S, Yingseree P, Yoshiike K, et al. Survival benefit from non-highly active antiretroviral therapy in a resource-constrained setting. *J Acquir Immune Defic Syndr* 2003;**32**:157–60.
16. Wichukchinda N, Nakayama EE, Rojanawiwat A, Pathipvanich P, Auwanit W, Vongsheree S, et al. Protective effects of IL4-589T and RANTES-28G on HIV-1 disease progression in infected Thai females. *AIDS* 2006;**20**:189–96.
17. Ministry of Public Health. *National guidelines for the clinical management of HIV infection in children and adults*. 6th ed. Thailand: Ministry of Public Health; 2000.
18. Tsuchiya N, Pathipvanich P, Yasuda T, Mukoyama Y, Rojanawiwat A, Matsubayashi T, et al. Demographic, socio-economic, behavioral and clinical factors predicting virologic failure with generic fixed-dose combination antiretroviral therapy before universal health insurance coverage in northern Thailand. *Southeast Asian J Trop Med Public Health* 2009;**40**:71–82.
19. Ledergerber B, Egger M, Erard V, Weber R, Hirschel B, Furrer H, et al. AIDS-related opportunistic illnesses occurring after initiation of potent antiretroviral therapy: the Swiss HIV Cohort Study. *JAMA* 1999;**282**:2220–6.
20. Egger M, May M, Chene G, Phillips AN, Ledergerber B, Dabis F, et al. Prognosis of HIV-1-infected patients starting highly active antiretroviral therapy: a collaborative analysis of prospective studies. *Lancet* 2002;**360**:119–29.
21. Manosuthi W, Chaovavanich A, Tansuphaswadikul S, Prasithsirikul W, Inthong Y, Chottanapund S, et al. Incidence and risk factors of major opportunistic infections after initiation of antiretroviral therapy among advanced HIV-infected patients in a resource-limited setting. *J Infect* 2007;**55**:464–9.
22. Saraceni V, King BS, Cavalcante SC, Golub JE, Lauria LM, Moulton LH, et al. Tuberculosis as primary cause of death among AIDS cases in Rio de Janeiro, Brazil. *Int J Tuberc Lung Dis* 2008;**12**:769–72.
23. Whalen CC, Nsubuga P, Okwera A, Johnson JL, Hom DL, Michael NL, et al. Impact of pulmonary tuberculosis on survival of HIV-infected adults: a prospective epidemiologic study in Uganda. *AIDS* 2000;**14**:1219–28.
24. Etard JF, Ndiaye I, Thierry-Mieg M, Gueye NF, Gueye PM, Laniece I, et al. Mortality and causes of death in adults receiving highly active antiretroviral therapy in Senegal: a 7-year cohort study. *AIDS* 2006;**20**:1181–9.
25. Ghate M, Deshpande S, Tripathy S, Nene M, Gedam P, Godbole S, et al. Incidence of common opportunistic infections in HIV-infected individuals in Pune, India: analysis by stages of immunosuppression represented by CD4 counts. *Int J Infect Dis* 2009;**13**:e1–8.
26. French N, Gray K, Watera C, Nakiyingi J, Lugada E, Moore M, et al. Cryptococcal infection in a cohort of HIV-1-infected Ugandan adults. *AIDS* 2002;**16**:1031–8.
27. Sriplung H, Parkin DM. Trends in the incidence of acquired immunodeficiency syndrome-related malignancies in Thailand. *Cancer* 2004;**101**:2660–6.
28. d'Arminio Monforte A, Sabin CA, Phillips A, Sterne J, May M, Justice A, et al. The changing incidence of AIDS events in patients receiving highly active antiretroviral therapy. *Arch Intern Med* 2005;**165**:416–23.
29. Losina E, Yazdanpanah Y, Deuffic-Burban S, Wang B, Wolf LL, Messou E, et al. The independent effect of highly active antiretroviral therapy on severe opportunistic disease incidence and mortality in HIV-infected adults in Cote d'Ivoire. *Antivir Ther* 2007;**12**:543–51.
30. Murdoch DM, Venter WD, Van Rie A, Feldman C. Immune reconstitution inflammatory syndrome (IRIS): review of common infectious manifestations and treatment options. *AIDS Res Ther* 2007;**4**:9.
31. French MA, Lenzo N, John M, Mallal SA, McKinnon EJ, James IR, et al. Immune restoration disease after the treatment of immunodeficient HIV-infected patients with highly active antiretroviral therapy. *HIV Med* 2000;**1**:107–15.

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Clinical Transplantation

Honoring the contract with our patients: outcome after repeated re-transplantation of the liver

Eguchi S, Soyama A, Mergental H, van den Berg AP, Scheenstra R, Porte RJ, Slooff MJH. Honoring the contract with our patients: outcome after repeated re-transplantation of the liver.
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Abstract: The aim of this study was to describe the outcome after repeated orthotopic liver re-transplantations (re-OLT) in a population of adults and children, and to determine whether such repeated re-transplantations are an effective treatment or should be considered futile. In a consecutive series of 867 patients, 628 adults and 239 children, who underwent OLT at the University Medical Center Groningen, 23 patients (2.7%), 10 adults and 13 children, underwent more than two re-transplantations of the liver between March 1979 and October 2008. All 23 patients had a second re-transplantation, and seven of them received a third transplant. The overall actuarial patient survival at 1, 5, and 10 yr after primary OLT was 96%, 87%, and 71%, respectively. The overall actuarial patient survival after the second re-OLT was 78%, 73%, and 67%, respectively. Sixteen patients (70%) survived long term. However, for the 23 repeated re-transplantation patients, 76 grafts were used. In a simulation calculation, it was shown that honoring the initial commitment to the 23 patients ultimately led to more surviving patients and less death than if treatment of the original patients was stopped after the first re-transplantation and the remaining grafts were allocated to other primary graft recipients.

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Key words: liver – multiple – repeated – re-transplantation

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The number of orthotopic liver transplantations (OLT) performed is still increasing worldwide each year because of a variety of reasons. The access to this treatment modality for patients with end-stage liver disease has increased as a result of the growing number of liver transplant centers in the world. In addition, the indications for transplantation have widened, and more elderly patients are accepted for transplantation (1, 2). Primary liver transplantation is still hampered by graft failure at a rate of 14–27%, 25–36%, and 38–47% at 1, 5, and 10 yr after OLT, as reported by the European Liver Transplant Registry and various individual centers (1–3). Because of the increasing number of primary transplantations, the need for re-transplantation (re-OLT) has also increased. Although the results of re-OLT are still inferior to those reported after primary OLT, several reports have published improving results of first re-OLTs during the last decade (3–5). Prognostic variables for survival after re-OLT have been identified, including recipient age, era of transplantation, UNOS status, number of previous transplantations, serum creatinine and bilirubin levels, cause of first graft failure, interval to re-OLT, and the donor risk index (4–9). This knowledge makes the proper selection of candidates for re-OLT possible and thereby prevents futile use of the available donor tissue pool. This evolution has contributed significantly to the acceptance of first re-OLTs, despite the limited donor pool.

Only a few reports are available regarding the outcome of second and third re-OLTs. Most of such repeated re-OLTs are reported in overall reviews of single center experiences and do not focus on the outcome of repeated transplantations in individual patients (7, 10, 11). Markman et al. (7) have reported that there were no one-yr

survivors in patients who received more than three grafts. Doyle et al. (12) also reported very poor survival rates after multiple re-OLT. Some individual centers were able to report better results. Pitre et al. (10) published the results of second re-OLTs in eight patients, five infants, and three adults, in 1997. They reported no survivors in three emergency cases, but all five elective cases (62%) survived; however, the survival was at the cost of substantial morbidity. Kumar et al. (11) reported five patients, one child and four adults, with second and third re-OLTs with a one-yr survival rate of 80%. Recently, Akpinar et al. (13) reported patient and graft survival rates at one yr of 72% and 56% after OLT of more than two grafts, with a perioperative mortality of 25%, while Marudanayagam et al. (14) reported that the five-yr survival after second re-transplantation was 40%. During the last decade, the donor shortage has not improved, and shortages are still universal. Therefore, it remains debatable whether the use of multiple grafts for individual recipients is justified (5, 6). For first re-OLT, when performed after selecting patients with prognostic indicators for success, this question is not relevant anymore because of acceptable survival results after the first re-OLT (3–6). However, for repeated re-OLTs, this question remains relevant. The aim of this paper was to report the results, in terms of survival, of repeated (second and third) re-OLTs in adults and children performed at the University Medical Center Groningen (UMCG) between March 1979 and October 2008.

Patients and methods

Multiple re-transplant patients were defined as patients who received more than two re-OLTs during the study period. Pediatric patients were defined as patients younger than 17 yr of age. During the study period, 867 patients, including 628 adults and 239 pediatric patients, underwent 1041 OLTs at the UMCG. In Table 1, an overview is given concerning the re-OLTs performed during that period. All cases of re-OLT were discussed before being listed by an internal review board composed of surgeons, (pediatric) hepatologists, anesthesiologists, and transplant coordinators with regard to the general status, performance status, renal function, infectious complications, and technical feasibilities of the re-OLT procedure. Patients with non-compliance, active infection outside the liver, or poor cardiac and pulmonary function were excluded for re-OLT.

As can be observed in Table 1, 144 (16.6%) of the 867 patients needed a first re-OLT. Twenty-three (2.7%) of the 867 patients (10 adults and 13 pediatric patients) underwent a second re-OLT and formed the study group. Seven (30.4%) of these 23 patients (four adults and three children) needed a subsequent third re-OLT. Multiple re-OLTs were performed in 10 (1.5%) of 628 adults compared to 13 (5.4%) of 239 children ($p = 0.004$).

The median follow-up of the patients since their primary OLT was 115 months (50–224 months) for adults and 112 months (8–204 months) for children.

Perioperative care

Only grafts from hemodynamically stable ABO identical or compatible brain dead donors with normal or near normal liver functions were accepted for re-OLT. Grafts were retrieved according to standard techniques. In case a graft needed to be reduced or split for re-OLT in a child, the criteria set by the Ville de Goyet were used for donor selection (15). The majority of the grafts were preserved in University of Wisconsin Solution, with a few exceptions before 1989, when the Euro Collins solution was used in our program. Grafts were implanted with the conventional or piggy back technique as reported previously by our group (16, 17).

Infection prevention (bacterial and viral) in patients with re-OLTs was essentially the same as

Table 1. Overall liver transplantation experience UMCG in the period March, 1979 till October, 2008

	Total		Adults		Children	
	Patients	Transplants	Patients	Transplants	Patients	Transplants
Total experience	867	1041	628	723	239	318
Re-transplantations						
First re-OLT	144 (16.6%)	144	82 (13.1%)	82	62 (26.0%)	62
Second re-OLT	23 (2.7%)	23	10 (1.5%)	10	13 (5.4%)	13
Third re-OLT	7 (0.8%)	7	3 (0.5%)	3	4 (2%)	4

re-OLT, orthotopic liver re-transplantations.