VARIABLES INFLUENCING ANTI-HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 NEUTRALIZING HUMAN MONOCLONAL ANTIBODY (NHMAB) PRODUCTION AMONG INFECTED THAIS

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Abstract. We conducted this study to determine the clinical variables associated with the production of human immunodeficiency virus type 1 (HIV-1) circulating recombinant form (CRF) 01_AE neutralizing human monoclonal antibodies (NhMAbs) using a hybridoma technique. This cross sectional study was performed in 20 asymptomatic HIV-1-infected Thais. Peripheral blood mononuclear cells (PBMCs) were obtained from each study participant and fused with SPYMEG cells. Culture supernatant collected from growing hybridomas was tested for neutralizing activity against HIV-1 CRF01_AE Env-recombinant viruses. Fifty hybridomas expressing anti-HIV-1 NhMAbs with strong neutralizing activity against at least 1 CRF01_AE Env-recombinant virus were found. A positive association between the numbers of hybridomas produced and the CD4 counts

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of study participants ($p = 0.019$) was observed. NhMAb-producing hybridomas with strong neutralizing activity were mostly found in participants diagnosed with HIV-1 infection within the previous 1 year. The HIV-1 viral load was not significantly correlated with the numbers of either established hybridomas or clones expressing anti-HIV-1 NhMAbs with strong neutralizing activity. To our knowledge, this is the first study of NhMAb-producing hybridomas obtained from HIV-1 CRF01_AE-infected populations identified by antibody binding to HIV-1 V3 loop peptide enzyme-linked immunosorbent assay (ELISA) or TRUGENE HIV-1 Genotyping Assay (HIV-1 pol sequence). It provides important criterion to select study participants with high CD4 counts who produce large numbers of hybridoma clones. The results are valuable for further studies related to neutralizing antibodies production and HIV-1 vaccine development.

Keywords: neutralizing human monoclonal antibody, HIV-1, CRF01_AE, CD4, hybridoma

INTRODUCTION

Both cellular and humoral immune responses occur after human immunodeficiency virus type 1 (HIV-1) infection. CD8+ T cells play a major role in the cell-mediated immune control of HIV-1 replication (Saez-Cirion *et al*, 2007), while neutralizing antibodies (NAbs) may play an important role in controlling infection. Neutralizing human monoclonal antibodies (NhMAbs) against epitopes of HIV-1 gp120 and gp41 play an important role not only in successfully blocking viral attachment to receptors expressed on CD4+ T cells, which further limit viral infection and replication (Wyatt and Sodroski, 1990; Lekkerkerker *et al*, 2004; van Montfort *et al*, 2007), but also in vaccine development (Burton *et al*, 2004). However, HIV-1 vaccine-induced antibodies have demonstrated limited protection against HIV-1 infection due to lack of neutralizing activities (Gilbert *et al*, 2005; The rgp120 HIV Vaccine Study Group, 2005; Pitisuttithum *et al*, 2006). A recent HIV-1 vaccine study conducted in Thailand demonstrated a small protective effect on the acquisition of infection. However, it did not affect HIV-1 viral load or CD4 counts among study participants who became HIV-1-infected during the course of the trial (Rerks-Ngarm *et al*, 2009).

Limited numbers of NhMAb have demonstrated potent neutralizing activity against diverse strains of HIV-1 (Buchacher *et al*, 1994; Trkola *et al*, 1996; Parren *et al*, 2001; Stiegler *et al*, 2001; Walker *et al*, 2009; Wu *et al*, 2010). IgG1 b12 was developed from the bone marrow of an asymptomatic HIV-1-infected individual (Burton *et al*, 1991, 1994) and was found to be protective against simian/human immunodeficiency virus (SHIV) infection in macaques after passive immunization (Parren *et al*, 2001). Antibodies 2G12 and 4E10 have been generated from peripheral blood mononuclear cells (PBMCs) of an HIV-1-infected volunteer using a cell fusion approach (Buchacher *et al*, 1994). 2G12 potently neutralized primary isolates of HIV-1 subtype B and some strains of subtype A (Buchacher *et al*, 1994; Trkola *et al*, 1996), while 4E10 strongly neutralized several subtypes of HIV-1, including A, B, C, D, F, G and CRF01_AE (Stiegler *et al*, 2001). Two additional NhMAbs,

PG9 and PG16, were obtained from an HIV-1 subtype A-infected donor using a short-term B cell culture. These two antibodies recognized epitopes expressed on trimeric gp120 envelope proteins and broadly neutralized a variety of viral subtypes (A, B, C, D, F, G, CRF01_AE, and CRF_AG) (Walker *et al*, 2009). Recently, VRC01 had been identified from an HIV-1 subtype B-infected subject using a computerized approach to design an antigenically resurfaced viral envelope which was specifically recognized by NhMAb at the CD4-binding site. VRC01 is another powerful NhMAb capable of neutralizing diverse subtypes of HIV-1 (A, B, C, D, G, CRF01_AE, CRF02_AG, and CRF07_BC) (Wu *et al*, 2010). Although HIV-1 subtype B only represents approximately 10% of the global prevalence (Taylor *et al*, 2008), HIV-1 subtype B infected-individuals are used as a major source for anti-HIV-1 NhMAb investigations (Burton *et al*, 1991; Buchacher *et al*, 1994; Humbert and Dietrich, 2006; Wu *et al*, 2010).

CRF01_AE is the predominant circulating form of HIV-1 in Thailand and Southeast Asia (Osmanov *et al*, 2002; Arroyo *et al*, 2010). In this cross-sectional study, using a hybridoma technique we demonstrated a significant association between CD4 counts and numbers of hybridomas established from HIV-1-infected Thai individuals. To our knowledge, this is the first report of anti-HIV-1 NhMAbs generated from HIV-1 CRF01_AE-infected cases, who were identified by antibody binding to HIV-1 V3 loop peptide enzyme-linked immunosorbent assay (ELISA) or TRUGENE HIV-1 Genotyping Assay (HIV-1 pol sequence). This data may be useful for developing further investigations related to either the establishment of anti-HIV-1 NhMAbs or vaccine development.

MATERIALS AND METHODS

Study participants

Asymptomatic Thai HIV-1-infected individuals who were anti-retroviral therapy naïve from Bamrasnaradura Infectious Diseases Institute and the Thai Red Cross AIDS Research Center were asked to participate in this study. The CD4 counts of all participants were obtained from the 6 month period prior to enrollment. To rule out the effect of immunization, only those who had not received any live attenuated vaccines up to 60 days prior to enrollment were included. Only those who had never participated in an HIV-1 vaccine trial were included in the study.

The B cell numbers of study participant were estimated using the formula:

Number of B cells = number of lymphocytes – number of CD4+T cells – number of CD8+T cells

This study was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University and the Institutional Review Boards of both the Bamrasnaradura Infectious Diseases Institute and the Faculty of Medicine, Chulalongkorn University. Written informed consent was obtained from all participants.

V3 loop peptide ELISA

The antibody binding to HIV-1 V3 loop peptide ELISA was conducted as previously described (Cheingsong-Popov *et al*, 1994). Briefly, a 96-well microplate was coated with B peptide (CTRPNNNTRKSIHLGPGKAWYTTGQIIGDIRQAH) (QCB, Hopkinton, MA), IDU B' peptide (CTRPNNNTRKSIHLGPGRAWYTTGQIIGDIRQAH) (Biosource International, Hopkinton, MA), and E peptide (CTRPSNNTRTSITIGPGQVFYRTGDIIGDIRKAY) (QCB, Hopkinton, MA),

which corresponded to HIV-1 subtype B and CRF01_AE, respectively, at a concentration of 0.5 mg/ml in 5% skim milk buffer for 14-18 hours at 2-8ºC. Plasma samples were diluted 1:100 in milk buffer and added to the coated microplate for 1 hour at 37ºC. Following incubation, the microplate was washed with phosphate-buffered saline containing 0.1% Tween-20. Bound antibodies were detected with goat anti-human immunoglobulin G specific peroxidase conjugate (EMD Biosciences, San Diego, CA). After washing, the color was developed with peroxidase substrate (KPL, Gaithersburg, MD). The optical density (OD) was measured at 405 nm, subtracting the background at 650 nm. A sample was determined to be HIV-1 subtype B or CRF01_AE when the corrected OD of one subtype was four times greater than the other subtype.

TRUGENE HIV-1 genotyping assay

Plasma HIV-1 ribonucleic acid (RNA) was extracted using an ultra-sensitive procedure by Roche; the Amplicor HIV-1 Monitor Test, v.1.5 (Roche Diagnostics, Branchburg, NJ). Extracted viral RNA was reverse transcribed to viral complementary deoxy-ribonucleic acid (cDNA) and sequenced using the TRUGENE HIV-1 Genotyping kit (Siemens Medical Solutions Diagnostics, Tarry Town, NY). Sequence data was analyzed and compared to the wild-type reference sequence using the OpenGene DNA Sequencing System (Siemens Medical Solutions Diagnostics, Tarry Town, NY). The HIV-1 serotype in the plasma sample was analyzed as previously described (Grant *et al,* 2003). The HIV-1 pol sequence data were submitted to GenBank (www.ncbi.nlm.nih.gov/genbank/) to acquire GenBank accession numbers for the nucleotide sequences.

Establishment of hybridomas

PBMCs were obtained from 15 ml of blood using density gradient centrifugation through ficoll-paque PLUS (GE Healthcare, Uppsala, Sweden) for 40 minutes at 560*g*. The PBMCs were then fused with the SPYMEG cells, which were used as fusion partner cells for establishing NhMAbs with polyethylene glycol (Roche, Mannheim, Germany) as previously described (Kubota-Koketsu *et al,* 2009; Setthapramote *et al,* 2012). After fusion, $1x10^4$ fused cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Grand Island, NY) supplemented with 15% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT) and hypoxanthine-aminopterin-thymidine (HAT) (Invitrogen, Grand Island, NY) in each well of the 96-well microplate at 37ºC with 5% carbondioxide (CO_2). A growing hybridoma was observed under microscope on day 14 post-fusion. Culture supernatant was collected from the growing hybridomas on days 14, 17, and 20 post-fusion and independently screened against CRF01_AE Env-recombinant viruses for neutralizing activity.

Preparation of recombinant viruses

Two proviral DNA segments, pNL-55PL1 and pNL-105PL3, were constructed in 2009, and contain CRF01_AE env genes (Genbank accession numbers EU743774 and EU743793, respectively) derived from HIV-1-infected Thai patients (Utachee *et al,* 2009a). The recombinant viruses generated from the constructs are both type R5, show high infectivity to U87.CD4.CCR5 cells, and are highly susceptible to pooled, HIV-1-positive patient plasma samples (ID50 >1,000). Both recombinant viruses are susceptible to anti-gp41 neutralizing human monoclonal antibody 4E10, but are resistant to anti-gp120 neu-

tralizing human monoclonal antibodies IgG1 b12 and 2G12, which were obtained from subtype B-infected patients (Utachee *et al*, 2009b). Stocks of viral supernatant were prepared by transfecting 293T cells with the proviral constructs using FuGENE HD transfection reagent (Roche, Mannheim, Germany) as previously described (Utachee *et al*, 2009b, 2010). At 48 hours post-transfection, the supernatant was centrifuged at 630*g* for 5 minutes. The viral titers were defined by measurement of HIV-1 Gag p24 antigen concentration in the viral supernatant samples using the HIV-1 p24 ELISA kit (BioAcademia, Osaka, Japan).

Neutralization assay

Twenty microliters undiluted culture supernatant was collected from growing hybridomas and pre-incubated with 80 l viral supernatant, NL-55PL1 and NL-105PL3, at a p24 antigen concentration of 2 nanograms (ng) at 37ºC for 30 minutes as previously described (Utachee *et al*, 2009b). Viral supernatant was pre-incubated with DMEM supplemented with 15% FBS and HAT in a minimum of 4 wells as a positive control to demonstrate the infectivity of CRF01_AE Env-recombinant viruses and evaluate the effect of culture medium components of recombinant viruses. Virus-free medium was employed as a negative control to evaluate the effect of the culture medium components on the U87.CD4.CCR5 cells. U87.CD4.CCR5 cells were cultured in the 96-well microplate at a density of $5x10^3$ cells/well in 100 l culture medium at 37ºC with 5% CO_2 for 24 hours prior to the neutralization assay. The U87.CD4.CCR5 cells were obtained from Drs HongKui Deng and Dan R Littman through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

Following incubation, 100 l hybridoma/virus supernatant was added to the cultures of U87.CD4.CCR5 cells and incubated at 37ºC with 5% CO_2. At 48 hours post-infection, luciferase activity in the lysates of viral-infected U87.CD4.CCR5 cells was assessed using the Steady Glo Luciferase assay kit (Promega, Madison, WI) with the Centro LB960 luminometer (Berthold Technologies, Bad Wildbad, Germany). Neutralizing activity was calculated as the percent reduction in relative luminescence units (RLUs) compared to the positive controls after subtracting the RLUs of the tested experiment. The average RLUs of the positive controls was considered as 100% of the neutralizing activity.

Percent RLUs reduction = [(Average RLUs of the positive control - RLUs of the tested experiment) / Average RLUs of the positive control] x 100

Cloning and large scale production of anti-HIV-1 NhMAb-producing hybridoma clone

A growing hybridoma with >70% cell confluence was peeled from the original well and diluted with DMEM supplemented with 15% FBS and HT supplement (Invitrogen, Grand Island, NY) to give 1 cell per well in the 96-well microplate. The diluted cells were cultured at 37ºC in 5% CO_2. When a growing single hybridoma clone covered >70% of the growing surface (>70% cell confluence), it was passaged into 3 wells of a 96-well microplate and cultured until it reached >70% cell confluence. Then, the growing single hybridoma clones were sub-cultured and recloned in one well of a 24-well plate using the same culture procedure. This cloning procedure was conducted two more times in two wells and one more time in four wells of a 24-well plate. When this was successfully completed,

the single hybridoma clones were further sub-cultured in a 6-well plate and 25- and 75-ml culture flasks. Following successful propagation of the single hybridoma clones, the cells were expanded and stored cryopreservatively.

Statistical analysis

The relationship between number of produced hybridomas and clinical variables of the study participants (CD4 counts, HIV-1 viral loads, time since HIV-1 diagnosis and the number of B cells) was assessed using a nonparametic Spearman's rank order correlation coefficient. Since the number of participants was small, they were grouped based on: 1). a CD4 count of 500 cells/ l, 2). an HIV-1 viral load of 10,000 copies/ml, and 3). 12 months since the diagnosis of having HIV. Differences in the number of hybridomas produced among the participant groups were resolved using a nonparametric Mann-Whitney *U* test. A two-tailed analysis ($p \leq 0.05$) was defined as significant. All statistical analyses were carried out using SPSS package, version 14 (SPSS, Chicago, IL). All illustrations were created using GraphPad Prism, version 5.03 (GraphPad Software, San Diego, CA).

RESULTS

Demographics of study participants

A total of 20 participants were enrolled in the study; 6 from the Bamrasnaradura Infectious Diseases Institute and 14 from the Thai Red Cross AIDS Research Center. The majority of study participants (65%) were male (Table 1). The median (range) age of participants was 29 (19-43) years old. Enrolled subjects were naïve to anti-retroviral therapy and did not show any clinical symptoms of AIDS-related diseases at the time of enrollment. The medians (range) CD4 count and plasma viral load were 572 (346-967) cells/ l and 29,144 (1,840-222,871) copies/ml, respectively. Thirteen subjects (65%) were enrolled in the study within 12 months of the diagnosis of HIV-1. The median (range) time since HIV-1 diagnosis at the time of enrollment was 2.25 (0.23-150.07) months. Fourteen participants for whom values were available had a median (range) B cell count of 711 (322-1,321) cells/ l (Table 1). No correlation between the CD4 count and time since diagnosis was found. As expected, CD4 counts were significantly negatively correlated with plasma HIV-1 viral loads ($r_s = -0.511$; $p = 0.021$). A significant positive association was seen between CD4 counts and B cell numbers ($r_s = 0.534$, $p = 0.049$).

HIV-1 serotyping

Using the HIV-1 subtype-specific V3 loop peptide ELISA, 16 out of the 20 participants (80%) were infected with CRF01_AE, 1 out of 20 was infected with subtype B and in the other 3 subjects, the infecting subtype could not be determined by the HIV-1 subtype-specific V3 loop peptide ELISA because the corrected ODs of both subtypes were comparable. Their subtypes were identified using the TRUGENE HIV-1 Genotyping assay. They were infected with HIV-1 CRF01_AE (Genbank accession numbers KF111717, KF111719 and KF111720, respectively). The participant who was identified as having HIV-1 subtype B with the HIV-1 subtype-specific V3 loop peptide ELISA, was confirmedly to be infected with subtype B by the TRUGENE HIV-1 Genotyping assay (Genbank accession numbers KF111718).

Of the 20 participants, 19 (95%) were infected with CRF01_AE and one was

Table 1
Demographics and laboratory values from study participants.

	Total	Male	Female
Number	20 (100%)	13 (65%)	7 (35%)
Age	29 (19-43)	29 (19-37)	29 (21-43)
CD4 counts (cells/ l)	572 (346-967)	571 (346-967)	572 (493-944)
Plasma viral load (copies/ l)	29,144 (1,840-222,871)	36,457 (3,880-222,871)	3,786 (1,840-92,895)
Time since diagnosis (months)	2.25 (0.23-150.07)	1.90 (0.23-46.50)	15.83 (1.67-150.07)
B cell numbers[a] (cells/ l)	711 (322-1,321)	573 (322-1,321)	872 (342-1,048)

Data is shown as medians with the range in parenthesis.
[a] Data obtained from 14 study participants.

Table 2
Various laboratory values among study subjects.

ID	HIV-1 serotypes	CD4 cell counts in cells/ l	B cell number in cells/ l	No. of hybridomas	No. of monoclones	No. hybridoma with ≥50% NT	
						Against 1 virus (%)	Against 2 viruses (%)
TMR01	CRF01_AE	825	N/A	100	85	0	0
TMR02	CRF01_AE	437	N/A	32	0	0	0
TMR03	CRF01_AE	493	N/A	38	0	0	0
TMR04	CRF01_AE	346	N/A	11	0	0	0
TMR05	CRF01_AE	566	N/A	288	253	0	0
TMR06	CRF01_AE	605	N/A	169	0	0	0
TMR07	CRF01_AE	967	1,321	123	130	12 (9.2)	1 (0.8)
TMR08	CRF01_AE	589	590	78	39	7 (17.9)	1 (2.6)
TMR09	CRF01_AE	571	322	78	174	16 (9.2)	0
TMR10	CRF01_AE[a]	944	807	36	90	0	0
TMR11	B[a]	701	1,106	90	104	1 (1.0)	0
TMR12	CRF01_AE	538	928	115	70	0	0
TMR13	CRF01_AE	568	342	120	273	9 (3.3)	4 (1.5)
TMR14	CRF01_AE[a]	606	1,048	233	0	0	0
TMR15	CRF01_AE	743	615	232	55	2 (3.6)	0
TMR16	CRF01_AE	583	452	124	238	1 (0.4)	0
TMR17	CRF01_AE	552	553	55	76	1 (1.3)	0
TMR18	CRF01_AE	542	437	27	0	0	0
TMR19	CRF01_AE[a]	572	872	66	109	1 (0.9)	0
TMR20	CRF01_AE	563	573	14	0	0	0
Total				2,029	1,696	50 (2.9)	6 (0.4)

No., number; NT, neutralizing activities; N/A, not applicable.
[a]HIV-1 subtype was resolved using TRUGENE HIV-1 Genotyping kit.

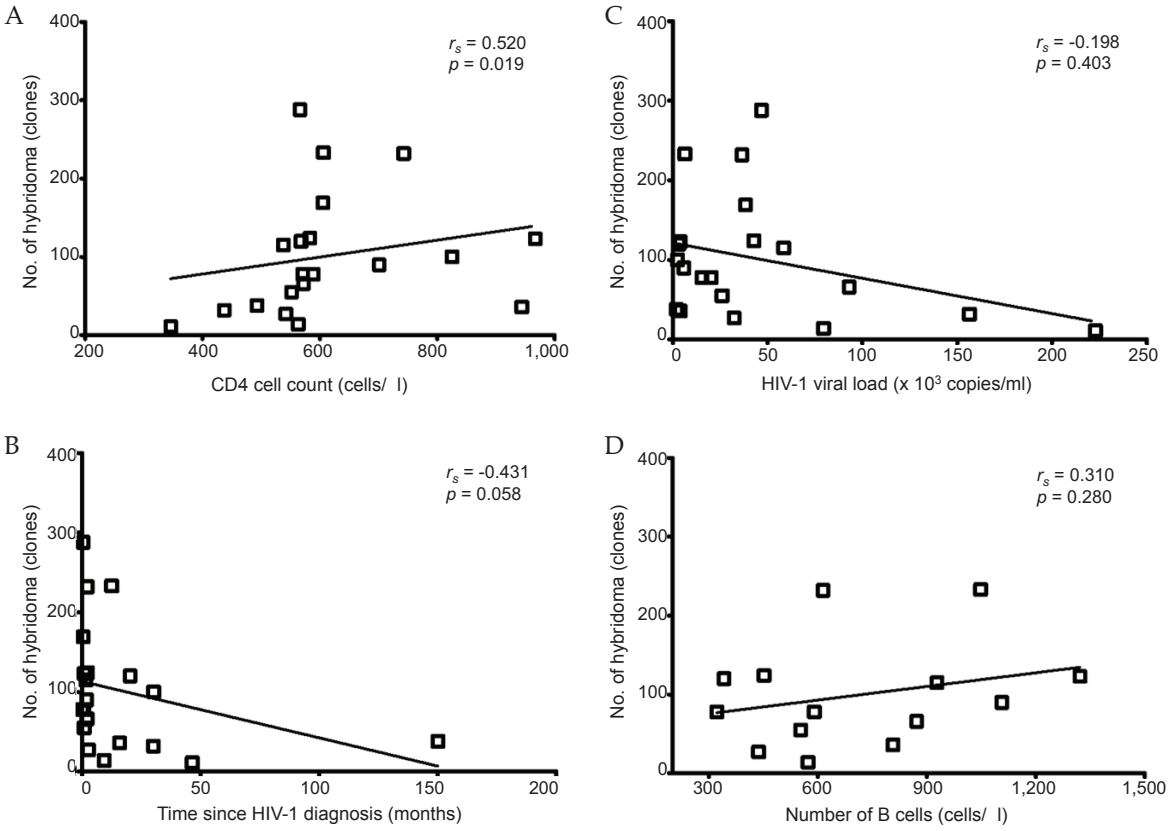

Fig 1–Correlation plots between number of hybridomas and (A) CD4 counts, (B) time since HIV-1 diagnosis, (C) HIV-1 viral load, and (D) B cell numbers.

infected with HIV-1 subtype B (Table 2).

Establishment of hybridomas producing anti-HIV-1 NhMAb

A total of 2,029 growing hybridomas (range : 11-288; median : 84) were visually observed under the microscope 14 days post-fusion. Nine subjects (45%) produced ≥100 hybridomas (Table 2). No differences in the numbers of hybridomas produced were seen between males and females.

Correlation between the number of established hybridomas and clinical variables in the study participants

We examined the relationship between the number of hybridoma produced and the clinical parameters of the study participants (CD4 counts, viral load levels, time since HIV-1 diagnosis, and B cell numbers) using Spearman's rank order correlation test. There was a statistically significant association (r_s = 0.520, p = 0.019, Fig 1A) between the number of hybridomas and the CD4 count. There was a trend toward a correlation between the number of hybridomas and the time since HIV-1 diagnosis (p = 0.058, Fig 1B). No correlation between the HIV-1 viral load or B cell numbers and the number of hybridomas produced was observed (Fig 1C and 1D).

The differences in the numbers of hybridomas produced in each group of study subjects was assessed by grouping

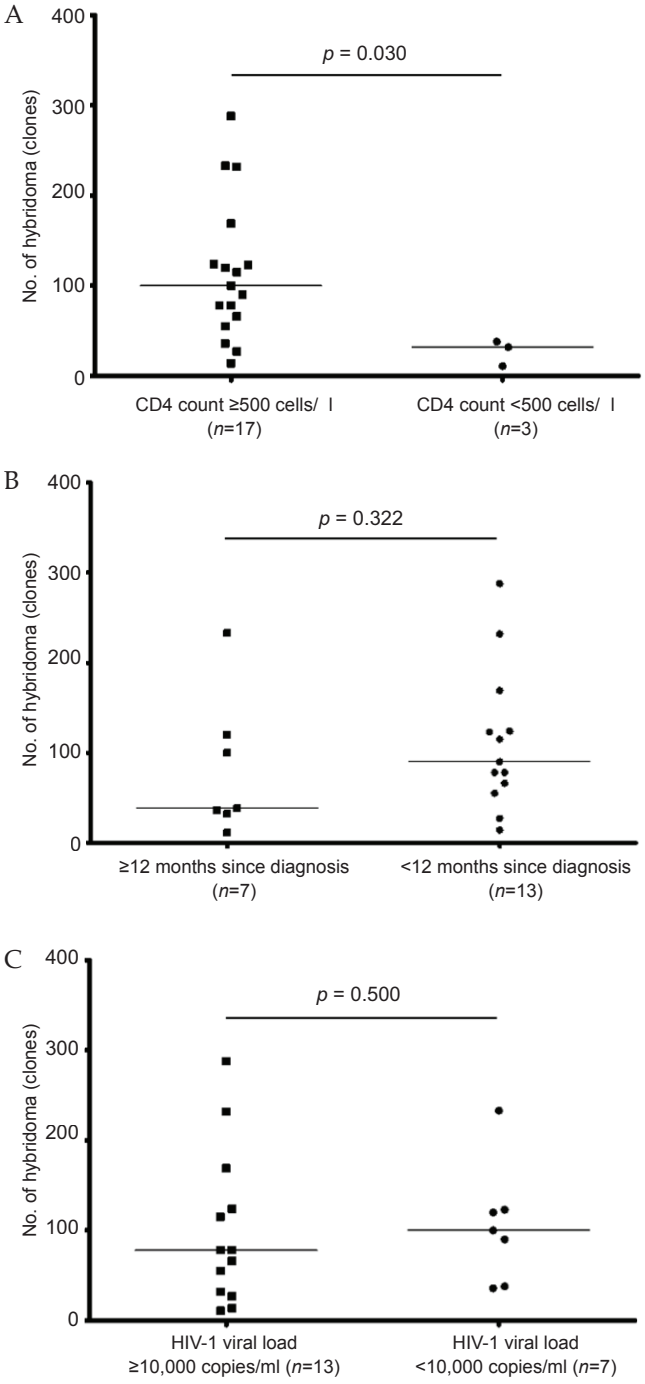

Fig 2–Comparison of number of hybridomas (A) among participants by CD4 cell counts, (B) by length of time diagnosed with having HIV-1 (C) by HIV-1 viral load. The median is shown as a back horizontal line.

study participants into 2 sub-sets based on 1) a CD4 count of 500 cells/ l, 2) a plasma viral load of 10,000 copies/ml, and 3) 12 months since HIV-1 diagnosis. A statistically significant difference in the number of hybridomas produced was seen between participants with a CD4 count < and those with ≥500 cells/ l (p = 0.030, Fig 2A). Subjects with a CD4 count ≥500 cells/ l produced 100 (14-288) hybridomas compared to subjects with a CD4 count <500 CD4/ l produced 32 (11-38) hybridomas. This phenomenon was not observed when study participants were stratified by time since HIV diagnosis or HIV-1 viral load (Fig 2B, 2C). Participants were not grouped by numbers of B cells because the results were not available for all participants.

These data suggest participants with higher CD4 counts produced higher numbers of hybridomas.

Neutralizing activity against CRF01_AE Env-recombinant viruses of established hybridoma

Cultured supernatant collected from all 2,029 identified hybridomas were screened 3 times (14, 17, and 20 days post-fusion) for neutralizing activity against 2 CRF01_AE Env-recombinant viruses, NL-55PL1 and NL-105PL3. These are the most sensitive of the 35 recombinant viruses to neutralization by pooled HIV-1-infected patient plasma (Utachee *et al*, 2009b). Both positive and negative controls showed CRF01_AE Env-recombinant viruses had high infectivity and culture medium components had no effect an CRF01_AE Env-recombinant viruses or U87.

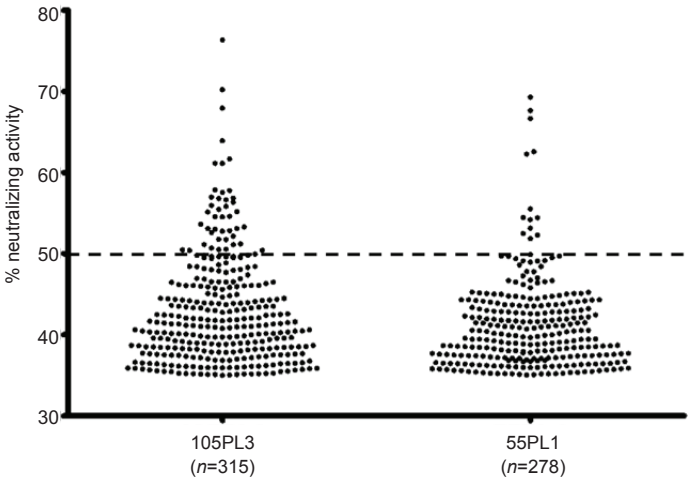

Fig 3–Dot plot demonstrating number of clones expressing ≥35% neutralizing activities against NL-105PL3 and NL-55PL1. Dashed line represents 50% neutralizing activity. 105PL3 = number of clones expressing ≥35% neutralizing activity against NL-105PL3; 55PL1 = number of clones expressing ≥35% neutralizing activity against NL-55PL1.

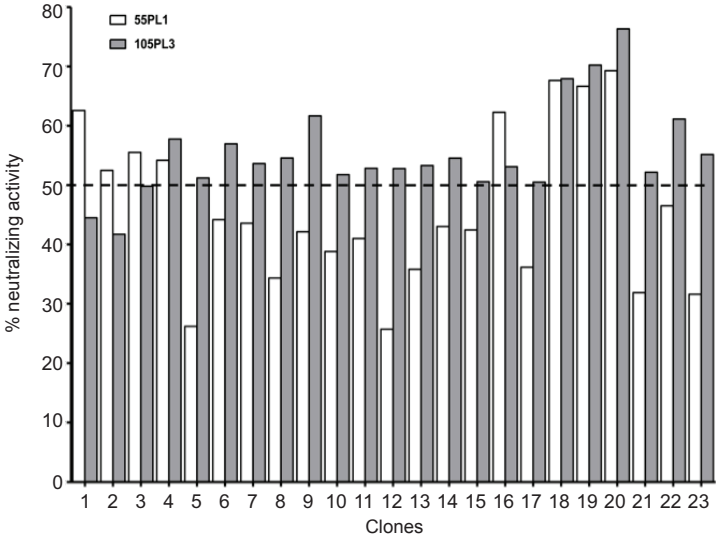

Fig 4–Neutralizing activity against NL-105PL3 and NL-55PL1 among 23 selected clones. Dashed line is 50% neutralizing activity. 105PL3=netralizing activity against NL-105PL3. 55PL1=neutralizing activity against NL 55PL1.

CD4.CCR5 cells (data not show).

One hundred eighteen hybridomas (5.8%) had the same or an increase in neutralizing activity (≥35% neutralizing activity) against at least 1 CRF01_AE Env-recombinant virus. They were next diluted in order to establish a single hybridoma clone producing anti-HIV-1 NhMAb. At 14 days post-dilution, 1,696 single hybridoma clones were identified. These were then re-assayed at 3 different times (days 14, 17, and 20 days post-diluton) for neutralizing activity against NL-55PL1 and NL-105PL3. Two hundred seventy eight (16.4%) and 315 (18.6%) single hybridoma clones had the same or in increase in neutralizing activity (≥35% neutralizing activity) against NL-55PL1 and NL-105PL3, respectively.

The majority of single hybridoma clones had <50% neutralizing activity against CRF01_AE Env-recombinant viruses, 265 of 278 (95.3%) and 272 of 315 (86.3%) single hybridoma clones had 35-49.9% neutralizing activity against NL-55PL1 and NL-105PL3, respectively (Fig 3). Overall, 50 single hybridoma clones (2.9%) expressed strong neutralizing activity (≥50% neutralizing activity) against at least 1 CRF01_AE Env-recombinant virus. Of these, 6 single hybridoma clones (0.4%) demonstrated

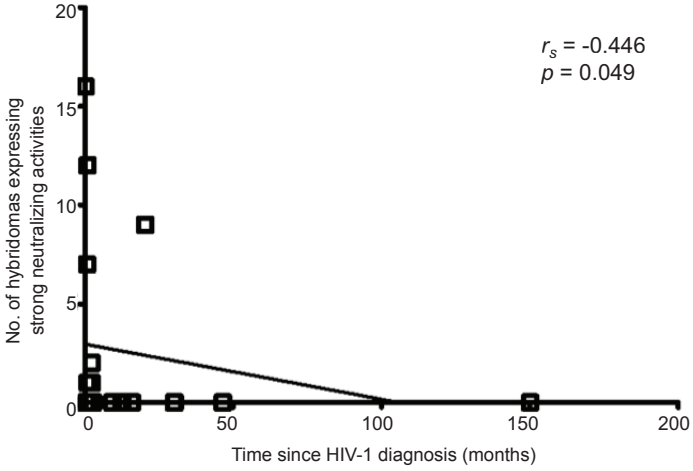

Fig 5–Correlation between the number of hybridomas expressing strong neutralizing activity against at least 1 CRF01_AE Env-recombinant virus by time since HIV-1 diagnosis.

strong neutralizing activity against both CRF01_AE Env-recombinant viruses (Table 2). Of the 50 single hybridoma clones expressing strong neutralizing activity, 23 (46.0%) were selected for expansion to establish large scale production of an anti-HIV-1 NhMAb-producing single hybridoma clone (Fig 4). Five (TM04, TM16, TM18, TM19, and TM20) of the six single hybridoma clones expressed strong neutralizing activity against two CRF01_AE Env-recombinant viruses; all of which were obtained from HIV-1 CRF01_AE-infected individuals, whose HIV-1 subtype was identified by antibody binding with the HIV-1 V3 loop peptide ELISA or HIV-1 pol sequence (TRUGENE HIV-1 Genotyping Assay). These were also selected for large scale production. One of six single hybridoma clones was lost during sub-culture.

These data demonstrate the successful establishment of hybridomas producing anti-HIV-1 NhMAb from Thai HIV-1 CRF01_AE-infected individuals, whose HIV-1 subtype was identified by antibody binding with the HIV-1 V3 loop peptide ELISA or HIV-1 pol sequence (TRUGENE HIV-1 Genotyping Assay).

Correlation between the number of single hybridoma clones espressing strong neutralizing activity and clinical variables of study participants

The association between the number of single hybridoma clones expressing strong neutralizing activity against CRF01_AE Env-recombinant virus and some clinical characteristics of the study participants was accessed using the Spearman rank order correlation test. There was a significant inverse correlation between time since HIV-1 diagnosis and the number of single hybridoma clones expressing strong neutralizing activity against at least 1 CRF01_AE Env-recombinant virus (r_s = -0.446, p = 0.049, Fig 5). A significant association was not observed with the CD4 count or HIV-1 viral load.

The data suggest an anti-HIV-1 NhMAb-producing single hybridoma clone with strong neutralizing activity against the CRF01_AE Env-recombinant virus is mostly found during the first year after HIV-1 diagnosis.

DISCUSSION

The serum of some HIV-1-infected patients contains potent, broad NAbs (Li *et al*, 2007); therefore, an understanding of how broad the NAbs are in infected individuals may provide valuable insight for the development of an effective HIV-1 vaccine. Several HIV-1 vaccines globally have been developed and evaluated for

their safety and efficacy in clinical trials. Two of three phase III HIV-1 vaccine clinical trials have been conducted in Thailand (International AIDS Vaccine Initiative, 2012). Type CRF01_AE accounts for approximately 80% of HIV-1-infected individuals in Thailand (Arroyo *et al*, 2010); hence, the establishment of anti-HIV-1 NhMAbs in CRF01_AE-infected individuals may provide valuable information for the development of an effective vaccine against HIV-1 strains in Thailand. The present study describes an important criterion for selecting study participants: high CD4 counts are important for the development of monoclones producing anti-HIV-1 NhMAb using hybridoma technology from clinically asymptomatic HIV-1 CRF01_AE-infected Thai subjects who are naïve to antiretroviral therapy. We found a strong positive correlation between the CD4 count and number of hybridomas. A significant inverse association was seen between the length of time since HIV-1 diagnosis and number of monoclones expressing strong neutralizing activity. No correlation was observed between the strength of neutralizing activity and other clinical parameters. No benefit exists for patient selection based on plasma viremia to obtain monoclones producing anti-HIV-1 NhMAb showing strong neutralizing activity, but it is better to select patients diagnosed with HIV-1 infection with high CD4 counts.

Ninety-five percent of our participants (19/20) were infected with HIV-1 CRF01_AE, which agrees with a previous cross-sectional study performed in Thailand (Arroyo *et al*, 2010) that reported approximately 80% of HIV-1 patients in Thailand are type CRF01_AE. The high percentage of patients with HIV-1 type CRF01_AE in our study may be due to the small sample size compared to the study by Arroyo *et al* (2010), which was conducted on approximately 400 samples. This study used antibody binding with the HIV-1 V3 loop peptide ELISA and the TRUGENE HIV-1 genotyping assay for HIV-1 serotyping. They did not detect the whole HIV-1 genome. Antibody binding to the HIV-1 V3 loop peptide ELISA detects antibodies specific to the V3 loop located on HIV-1 env glycoprotein, while the TRUGENE HIV-1 genotyping assay detects a genome sequence in the pol region. They demonstrated good accuracy, reliability and reproducibility with HIV-1 subtyping (Cheingsong-Popov *et al*, 1994; Kuritzkes *et al*, 2003). Of the 19 HIV-1 CRF01_AE-infected participants in our study, 9 generated clones expressing strong neutralizing activity against at least 1 CRF01_AE Env-recombinant virus. Interestingly, one monoclone with strong neutralizing activity was produced by a participant, who was infected with HIV-1 subtype B. This finding shows the possibility of anti-HIV-1 NhMAb expressing cross-reactivity; however, an additional confirmatory experiment should be performed to prove cross reactivity of this anti-HIV-1 NhMAb against other strains of HIV-1.

The HIV-1 produced in cell culture supernatant or in a patient's plasma may be quite heterogeneous, and only a small part of the virus is infectious, whereas the remaining viruses are non-infectious (Kameoka M, personal communication). In contrast, the recombinant viruses used in this study were produced from 293T cells transfected with proviral constructs; therefore, the produced viruses were quite homogenous and showed high infectivity (Utachee *et al*, 2009a, 2010).

We found a significant positive relationship between the number of hybridomas and the CD4 count in this study.

When participants were divided into 2 groups based on a CD4 count of 500 cells/ l, which the United States Centers for Disease Control and Prevention (CDC) has used as criteria for categorizing HIV-1-infected individuals as a non-immune deficiency HIV-1-infected patient (Centers for Disease Control and Prevention, 1992); the number of hybridomas produced in participants with a CD4 cell count ≥500 cells/ l was significantly higher than in those with lower CD4 counts. This may be explained by our finding of a significant positive correlation between the CD4 cell count and the numbers of B cells, as well as the observations from other studies that an effective CD4 T-cell response played an important role in an effective B-cell response (Chirmule *et al*, 1992; Moir *et al*, 2003). High CD4 counts may indirectly indicate a high B-cell response which could result in large numbers of hybridomas producing antibodies. Since no correlation between the number of B cells and the number of hybridomas produced was identified, we believe only the CD4 counts played an important influence on the production of hybridomas. When comparing the number of monoclones expressing strong neutralizing activity between groups of volunteers with a CD4 count < 500 cells/ l and ≥500 cells/ l, no differences were found; this agrees with other studies which reported no correlation between declining CD4 counts in HIV-1-infected patients, and the generation of potent, broad anti-HIV-1 NhMAbs (Kelly *et al*, 2005; Rodriguez *et al*, 2007; McLinden *et al*, 2012).

The present study demonstrated no relationship between the time since HIV-1 diagnosis and number of hybridomas produced; however, there was a significant inverse association between the time since HIV-1 diagnosis and the number of monoclones expressing strong neutralizing activity. When study participants were grouped into 2 subsets based on <12 months or ≥12 months since HIV-1 diagnosis, participants who had been diagnosed with HIV-1 for <12 months produced larger numbers of clones with strong neutralizing activity than those who had been diagnosed with HIV-1 ≥12 months previously (Fig 2B). This observation could be explained by the subject who had HIV-1 infection for a shorter time still had high CD4 counts, which correlated with large numbers of hybridomas produced. This could result in more opportunities to generate more monoclones expressing strong neutralizing activity. Some previous studies reported the development of a potent NAb response against HIV-1 within the first year of infection (Richman *et al*, 2003; Gray *et al*, 2007; Mikell *et al*, 2011), but other studies reported a high level of NAb response in chronically HIV-1-infected individuals (Deeks *et al*, 2006; Euler *et al*, 2010; Mikell *et al*, 2011).

In the present study, time since diagnosis might not equate to time of infection, because some people who had recently been diagnosed might have had the infection for years and had low CD4 counts (Ananworanich J, personal communication). It could be possible some participants enrolled in the present study might be infected with HIV-1 for years. This might be a reason why our findings were discordant with previous reports. In this study, the time since diagnosis did not influence the production of hybridomas but might have played a role in the magnitude of the NAb response.

No correlation was seen between the HIV-1 viral load and the number of hybridomas, which is in agreement with a previous report (Aasa-Chapman *et al*,

2004). However, another study found a significant association between persistently low viral loads (100-10,000 copies/ml) and the presence of broad NAbs in plasma (Sajadi *et al*, 2011). For this reason, we divided our participants into 2 groups based on a plasma viremia of 10,000 copies/ml. Although no significant differences in the number of hybridomas produced or the number of clones expressing strong neutralizing activity was seen between the 2 groups, it is possible the participants who had HIV-1 viremia <10,000 copies/ml could produce larger numbers of hybridomas and clone producing anti-HIV-1 NhMAbs expressing strong neutralizing activity than those who had an HIV-1 viral load ≥10,000 copies/ml. This observation could be due to impaired function of B cells among HIV-1-infected individuals with high HIV-1 viral loads leading to reduced antibody responses (Moir *et al*, 2001; Moir and Fauci, 2009). Other studies have found an association between plasma viral load and the number of NAbs (Rodriguez *et al*, 2007; Piantadosi *et al*, 2009). The discordance with other published reports could be explained by either the small sample size in this study or the narrow plasma viral load range in the subjects recruited.

The following shortcomings in our study must be considered. First, our study was designed to detect strong neutralizing activity (≥50% neutralizing activities) of NhMAbs obtained from the culture supernatant of hybridomas, which is different from studies that investigated the activities of NAbs contained in the patient's serum (Subbramanian *et al*, 2002; Gray *et al*, 2007; Rodriguez *et al*, 2007; Piantadosi *et al*, 2009; McLinden *et al*, 2012). Second, our study did not investigate neutralizing activity against other strains of HIV-1. This information is important to demonstrate the breadth of the activities of our anti-HIV-1 NhMAbs, which we could not include in the present study. Third, we did not perform antibody isotype or antigen epitope mapping studies.

To our knowledge, this is the first investigation of anti-HIV-1 NhMAbs obtained from asymptomatic HIV-1 CRF01_AE-infected Thai individuals, in whom their HIV-1 subtype was identified by antibody binding with the HIV-1 V3 loop peptide ELISA or HIV-1 pol sequence (TRUGENE HIV-1 Genotyping Assay). We also found a direct correlation between number of hybridomas and the CD4 count. Time since HIV-1 diagnosis was significantly inversely correlated to the number of clones expressing strong neutralizing activity against CRF01_AE Env-recombinant viruses. We present critical criteria for selecting study participants who have high CD4 counts to produce large numbers of hybridoma clones expressing neutralizing activity against HIV-1. Further studies of this monoclonal antibody and its relationship to HIV-1 vaccine development are warranted.

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REFERENCES

Aasa-Chapman M, Hayman A, Newton P, *et al*. Development of the antibody response in acute HIV-1 infection. *AIDS* 2004; 18: 371-81.

Arroyo M, Phanuphak N, Krasaesub S, *et al*. HIV type 1 molecular epidemiology among high-risk clients attending the Thai Red Cross Anonymous Clinic in Bangkok, Thailand. *AIDS Res Hum Retroviruses* 2010; 26: 5-12.

Buchacher A, Predl R, Strutzenberger K, *et al*. Generation of human monoclonal antibodies against HIV-1 proteins; electrofusion and epstein-barr virus transformation for peripheral blood lymphocyte immortalization. *AIDS Res Hum Retroviruses* 1994; 10: 359-69.

Burton D, Barbas C, Persson M, Koenig S, Chanock R, Lerner R. A large array of human monoclonal antibodies to type 1 human immunodeficiency virus from combinatorial libraries of asymptomatic seropositive individuals. *Proc Natl Acad Sci USA* 1991; 88: 10134-7.

Burton D, Desrosiers R, Doms R, *et al*. HIV vaccine design and the neutralizing antibody problem. *Nat Immunol* 2004; 5: 233-6.

Burton D, Pyati J, Koduri R, *et al*. Efficient neutralization of primary isolates of HIV-1 by a recombinant human monoclonal antibody. *Science* 1994; 266: 1024-7.

Centers for Disease Control and Prevention. 1993 revised classification system for HIV infection and expanded surveillance case definition for AIDS among adolescents and adults. *MMWR Recomm Rep* 1992; 41: 1-19.

Cheingsong-Popov R, Lister S, Callow D, Kaleebu P, Beddows S, Weber J. Serotyping HIV type 1 by antibody binding to the V3 Loop: relationship to viral genotype. *AIDS Res Hum Retroviruses* 1994; 10: 1379-86.

Chirmule N, Oyaizu N, Kalyanaraman V, Pahwa S. Inhibition of normal B-cell function by human immunodeficiency virus envelope glycoprotein, gp120. *Blood* 1992; 79: 1245-54.

Deeks S, Schweighardt B, Wrin T, *et al*. Neutralizing antibody responses against autologous and heterologous viruses in acute versus chronic human immunodeficiency virus (HIV) infection: evidence for a constraint on the ability of HIV to completely evade neutralizing antibody responses. *J Virol* 2006; 80: 6155-64.

Euler Z, van Gils M, Bunnik E, *et al*. Cross-reactive neutralizing humoral immunity does not protect from HIV type 1 disease progression. *J Infect Dis* 2010; 201: 1045-53.

Gilbert P, Peterson M, Follmann D, *et al*. Correlation between immunologic responses to a recombinant clycoprotein 120 vaccine and incidence of HIV-1 infection in a phase 3 HIV-1 preventive vaccine Trial. *J Infect Dis* 2005; 191: 666-77.

Grant R, Kuritzkes D, Johnson V, *et al*. Accuracy of the TRUGENE HIV-1 genotyping kit. *J Clin Microbiol* 2003; 41: 1586-93.

Gray E, Moore P, Choge I, *et al*. Neutralizing antibody responses in acute human immunodeficiency virus type 1 subtype C infection. *J Virol* 2007; 81: 6187-96.

Humbert M, Dietrich U. The role of neutralizing antibodies in HIV infection. *AIDS Rev* 2006; 8: 51-9.

International AIDS Vaccine Initiative (IAVI).

Database of vaccine candidates in clinical trials. New York: IAVI, 2012. [Cited 2012 Aug 3]. Available from: URL: http://www.iavireport.org/ TrialsDatabase/Pages/Results.aspx?searchid=4f4cdd96-f723-4679-8e27-4ad68b8ea5ed

Kelly H, Urbanski M, Burda S, *et al*. Neutralizing antibody patterns and viral escape in HIV-1 non-B subtype chronically infected treatment-naive individuals. *Hum Antibodies* 2005; 14: 89-99.

Kubota-Koketsu R, Mizuta H, Oshita M, *et al*. Broad neutralizing human monoclonal antibodies against influenza virus from vaccinated healthy donors. *Biochem Biophys Res Commun* 2009; 387: 180-5.

Kuritzkes D, Grant R, Feorino P, *et al*. Performance characteristics of the TRUGENE HIV-1 genotyping kit and the opengene DNA sequencing system. *J Clin Microbiol* 2003; 41: 1594-9.

Lekkerkerker A, Ludwig I, van Vliet S, van Kooyk Y, Geijtenbeek T. Potency of HIV-1 envelope glycoprotein gp120 antibodies to inhibit the interaction of DC-SIGN with HIV-1 gp120. *Virology* 2004; 329: 465-76.

Li Y, Migueles S, Welcher B, *et al*. Broad HIV-1 neutralization mediated by CD4-binding site antibodies. *Nat Med* 2007; 13: 1032-4.

McLinden R, Paris R, Polonis V, *et al*. Association of HIV neutralizing antibody with lower viral load after treatment interruption in a prospective trial (A5170). *AIDS* 2012; 26: 1-9.

Mikell I, Sather D, Kalams S, Altfeld M, Alter G, Stamatatos L. Characteristics of the earliest cross-neutralizing antibody response to HIV-1. *PLoS Pathog* 2011; 7: e1001251.

Moir S, Fauci A. B cells in HIV infection and disease. *Nat Rev Immunol* 2009; 9: 235-45.

Moir S, Malaspina A, Ogwaro K, *et al*. HIV-1 induces phenotypic and functional perturbations of B cells in chronically infected individuals. *Proc Natl Acad Sci U S A* 2001; 98: 10362-7.

Moir S, Ogwaro K, Malaspina A, *et al*. Perturbations in B cell responsiveness to CD4+ T cell help in HIV-infected individuals. *Proc Natl Acad Sci USA* 2003; 100: 6057-62.

Osmanov S, Pattou C, Walker N, Schwardlaender B, Esparza J, WHO-UNAIDS Network for HIV Isolation and Characterization. Estimated global distribution and regional spread of HIV-1 genetic subtypes in the year 2000. *J Acquir Immune Defic Syndr* 2002; 29: 184-90.

Parren P, Marx P, Hessell A, *et al*. Antibody protects macaques against vaginal challenge with a pathogenic R5 simian/human immunodeficiency virus at serum levels giving complete neutralization in vitro. *J Virol* 2001; 75: 8340-7.

Piantadosi A, Panteleeff D, Blish C, *et al*. Breadth of neutralizing antibody response to human immunodeficiency virus type 1 is affected by factors early in infection but does not influence disease progression. *J Virol* 2009; 83: 10269-74.

Pitisuttithum P, Gilbert P, Gurwith M, *et al*. Randomized, double-blind, placebo-controlled efficacy trial of a bivalent recombinant glycoprotein 120 HIV-1 vaccine among injection drug users in Bangkok, Thailand. *J Infect Dis* 2006; 194: 1661-71.

Rerks-Ngarm S, Pitisuttithum P, Nitayaphan S, *et al*. Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. *N Engl J Med* 2009; 361: 2209-20.

Richman D, Wrin T, Little S, Petropoulos C. Rapid evolution of the neutralizing antibody response to HIV type 1 infection. *Proc Natl Acad Sci USA* 2003; 100: 4144-9.

Rodriguez S, Sarr A, MacNeil A, *et al*. Comparison of heterologous neutralizing antibody responses of human immunodeficiency virus type 1 (HIV-1)- and HIV-2-infected Senegalese patients: distinct patterns of breadth and magnitude distinguish HIV-1 and HIV-2 infections. *J Virol* 2007; 81: 5331-8.

Saez-Cirion A, Lacabaratz C, Lambotte O, *et al*. HIV controllers exhibit potent CD8 T

cell capacity to suppress HIV infection ex vivo and peculiar cytotoxic T lymphocyte activation phenotype. *Proc Natl Acad Sci USA* 2007; 104: 6776-81.

Sajadi M, Guan Y, DeVico A, *et al*. Correlation between circulating HIV-1 RNA and broad HIV-1 neutralizing antibody activity. *J Acquir Immune Defic Syndr* 2011; 57: 9-15.

Setthapramote C, Sasaki T, Puiprom O, *et al*. Human monoclonal antibodies to neutralize all dengue virus serotypes using lymphocytes from patients at acute phase of the secondary infection. *Biochem Biophys Res Commun* 2012; 423: 867-72.

Stiegler G, Kunert R, Purtscher M, *et al*. A potent cross-clade neutralizing human monoclonal antibody against a novel epitope on gp41 of human immunodeficiency virus type 1. *AIDS Res Hum Retroviruses* 2001; 17: 1757-65.

Subbramanian R, Xu J, Toma E, *et al*. Comparison of human immunodeficiency virus (HIV)-specific infection-enhancing and inhibiting antibodies in AIDS patients. *J Clin Microbiol* 2002; 40: 2141-6.

Taylor B, Sobieszczyk M, McCutchan F, Hammer S. The challenge of HIV-1 subtype diversity. *N Engl J Med* 2008; 358: 1590-602.

The rgp120 HIV Vaccine Study Group. Placebo-controlled phase 3 trial of a recombinant glycoprotein 120 vaccine to prevent HIV-1 infection. *J Infect Dis* 2005; 191: 654-65.

Trkola A, Purtscher M, Muster T, *et al*. Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1. *J Virol* 1996; 70: 1100-8.

Utachee P, Jinnopat P, Isarangkura-na-ayuthaya P, *et al*. Genotypic characterization of CRF01_AE env genes derived from human immunodeficiency virus type 1-infected patients residing in central Thailand. *AIDS Res Hum Retroviruses* 2009a; 25: 229-36.

Utachee P, Jinnopat P, Isarangkura-na-ayuthaya P, *et al*. Phenotypic studies on recombinant human immunodeficiency virus type 1 (HIV-1) containing CRF01_AE env gene derived from HIV-1-infected patient, residing in central Thailand. *Microbes Infect* 2009b; 11: 334-43.

Utachee P, Nakamura S, Isarangkura-na-ayuthaya P, *et al*. Two N-linked glycosylation sites in the V2 and C2 regions of human immunodeficiency virus type 1 CRF01_AE envelope glycoprotein gp120 regulate viral neutralization susceptibility to the human monoclonal antibody specific for the CD4 binding domain. *J Virol* 2010; 84: 4311-20.

van Montfort T, Nabatov A, Geijtenbeek T, Pollakis G, Paxton W. Efficient capture of antibody neutralized HIV-1 by cells expressing DC-SIGN and transfer to CD4+ T lymphocytes. *J Immunol* 2007; 178: 3177-85.

Walker L, Phogat S, Chan-Hui P, *et al*. Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target. *Science* 2009; 326: 285-9.

Wu X, Yang Z, Li Y, *et al*. Rational design of envelope identifies broadly neutralizing human monoclonal antibodies to HIV-1. *Science* 2010; 329: 856-61.

Wyatt R, Sodroski J. The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. *Science* 1990; 280: 1884-8.