to mitochondrial depletion and dysfunction[8]; mainly stavudine (d4T), but also zidovudine (AZT) and didanosine (ddI) are strongly related to LA.[9,10] In previous *in vitro* and clinical studies, newer nucleoside and nucleotide agents, such as lamivudine (3TC), emtricitabine, abacavir, and tenofovir, appear to be much weaker inhibitors of mitochondrial DNA polymerase-γ or other mitochondrial functions, and appear to be associated with a lower risk of events thought to be related to mitochondrial toxicity.[11] However, a certain degree of mitochondrial toxicity is still present and will probably still affect HIV patients under ART, although to a lesser extent.[12–14] In fact, switch studies from d4T to other NRTIs and complete switch off of thymidine analogs showed modest even if consistent increases in limb fat.[15–17]

The pathogenesis of lipohypertrophy appears to be multifactorial, with age, sex, HIV itself, immune depression, and duration and type of ART related to its appearance.[18–20] PIs were suggested to be involved in lipohypertrophy; however, visceral fat accumulation also occurs in the absence of PIs.[21,22] A compartment-specific effect of mitochondrial toxicity within the adipose tissue may be related to lipohypertrophy[23] as well as dysregulation of fatty acid metabolism and altered expression of adipokines.[24–26]

However, LA and lipohypertrophy do not occur in all treated patients, and there is a very large degree of interindividual variability in the timing of emergence and severity of symptoms. A study of identical twins showed that genetic factors are involved in the accumulation of visceral adipose tissue.[27] These data suggest that host genetic factors may play a role, and inherited predispositions may have a significant influence on the appearance of LD and metabolic alterations as well as on the viroimmunological response to the drugs.

Therefore, host genetic polymorphisms may have a significant effect on the response to ART in terms of metabolism and immune response.[28] A study of Italian HIV patients suggested that single nucleotide polymorphisms (SNPs) in genes involved in apoptosis and lipid metabolism mediate the development of LA.[29] In particular, the TT and CT genotypes at position −455 of the *Apolipoprotein C3* (*ApoC3*) gene (*ApoC3* T–455C, rs2854116), nonsynonymous (W to R) CC and CT genotypes at codon 64 of the *Beta3 adrenergic receptor* (*ARβ3*) gene (*ARβ3* Tcod64C, rs4994), and the AA genotype at position −670 of the *Fas* gene (*Fas* A–670G, rs1800682) were associated with increased incidence rates of LA.[29] ApoC3 protein, localized mainly in very low-density lipoprotein and high-density lipoprotein (HDL), is involved in fat metabolism and may delay the catabolism of triglyceride-rich particles. ApoC3 inhibits lipoprotein lipase and hepatic lipase and decreases the chylomicrons in hepatic cells.[30,31] ARβ3 protein, expressed mainly in visceral adipose tissue,[32,33] contributes to both lipolysis and the delivery of free fatty acids. *Fas* is the main gene that controls cell death by inducing apoptosis. A previous study indicated that Fas and Fas ligand influenced the immune reconstitution induced by ART.[34]

In Thailand, the National Access to Antiretroviral Care program started in 2002 using standard regimens with GPO-VIR (a combination of d4T, 3TC, and nevirapine) as the first line of ART. The present study was performed to determine whether these genetic polymorphisms found in the Italian cohort also mediate the emergence of LA or LD in HIV-infected patients starting ART in Thailand in consideration of the relevance of these side effects in the currently used regimens and of the differences in ethnicity, alimentation, and way of life of the Thai population.

Materials and Methods

Study design

HIV-1-infected individuals receiving ART at Bamrasnaradura Infectious Diseases Institute, Nonthaburi, Thailand were enrolled in this study. LA was defined as the presence of fat loss at any site of the body (face, arms, legs, or buttocks). LD was defined as LA combined with fat accumulation diagnosed at the same time. Fat accumulation was defined as the presence of at least one alteration, such as buffalo hump, or fat accumulation in the breast, abdomen, neck, or lipomas. To qualify, the alteration had to be recognized by both the patient and the physician.[35] Most patients who developed LA or LD changed regimen for those without d4T. A 200 μl sample of whole blood was collected from each patient and stored at −20°C until DNA extraction. The genotypes of *ApoC3* −455, *ARβ3* cod64, and *Fas* −670 were compared between patients who developed LA or LD and those who did not. Patients' age, gender, transmission route, CD4 counts at pre-ART and at 1 year post-ART (cells/μl), drug regimens, and duration of therapy were obtained from their medical records. Lipid profile data were obtained from patients' records of serum biochemical tests. Blood for this test was collected when patients were in the fasting state; however, not all patients had serum biochemical test data as the test incurs extra costs for patients. All participants signed an informed consent form. The study was approved by the institutional ethical committees at the Bamrasnaradura Infectious Diseases Institute and Department of Disease Control, Ministry of Public Health, Thailand.

DNA extraction

Genomic DNA was extracted from 200 μl of each whole blood sample using a QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.[36]

Genotyping

Genotyping of *Fas* gene promoter polymorphism, A–670G (rs1800682). Polymerase chain reaction (PCR) and restriction fragment length polymorphism analysis for *Fas* A–670G, modified from a previously published technique,[37] was conducted with the primers 5′-CTACCTAAGAGCTATCTA CCGTTC-3′ and 5′-GGCTGTCCATGTTGTGGCTGC-3′. The PCR product was digested with the restriction enzyme *Mva*I (Roche Biochemicals, Mannheim, Germany) at 37°C overnight. Two polymorphic alleles could be distinguished on the basis of restriction length polymorphisms (188 bp for G and 232 bp for A).

Genotyping of *ApoC3* gene promoter polymorphism, T–455C (rs2854116). TaqMan SNP assay was performed using the StepOne real-time PCR system (Applied Biosystems, Foster City, CA) with primers (5′-GAGCTCAGCCCCTG TAACCAG-3′ and 5′-ACACAGCCTGGAGTAGAGGG-3′) and TaqMan MGB probes (5′-VIC-CTCCAAACACCCC-MGB-3′ for the C allele and 5′-FAM-TTTACTCCAAA CATCC-MGB-3′ for the T allele).

Genotyping of *ARβ3* gene coding polymorphism, Tcod64C (rs4994). TaqMan SNP assay was performed with a TaqMan SNP assay kit (C___2215549_20; Applied Biosystems).

Statistical analysis

Person-year analysis was conducted to investigate a single endpoint: the incidence of LA or LD. If the patient was diagnosed as both LA and LD, the earlier date of the first diagnosis was used as the endpoint. We focused on the time to the occurrence of the first event. Person-years at risk were calculated from the start date of ART until the last available follow-up or development of the event, whichever occurred first. The Poisson regression univariate model was used to determine whether the genetic polymorphisms as well as patients' age, gender, transmission route, CD4 counts at pre-ART and at 1 year post-ART, and starting drug regimes were predictors of LA or LD. Associations were expressed as incidence rate ratio (IRR) and corresponding 95% confidence interval (CI). Factors showing significant association with LA or LD were then included as covariates in the Poisson regression multivariate model to adjust for their possible confounding effects. Starting drug regimens were grouped into either d4T-containing regimens or other backbone regimens. Continuous variables were compared using the Wilcoxon signed rank test for paired samples and Kruskal–Wallis test for unpaired samples. A variable was considered significant at $p<0.05$.

Results

Incidences of LA and/or LD in HIV-1-infected Thai patients undergoing ART

A total of 829 HIV-1-infected individuals who had started ART were enrolled in this study at the Bamrasnaradura Infectious Diseases Institute, Nonthaburi, Thailand, from April 2008 to August 2009. Patient demographic data are shown in Table 1. The study population consisted of 478 males (57.7%) and 351 females (42.3%). Most of the patients contracted infection through sexual contact. Almost half of the patients started ART immediately after being diagnosed with HIV infection. Median CD4 count per microliter pre-ART was 45 and that post-ART (after 1 year) was 210.5. The mean follow-up period was 4.85 years (SD=2.42).

Table 1. Demographic Data of All the Patients

Demographic data	n=*829*
Mean age (SD) years	41.12 (7.77)
Male (%)	478 (57.66)
Transmission	
Sexual (%)	806 (97.23)
Intravenous drug user (%)	8 (0.97)
Unknown (%)	15 (1.81)
Median baseline CD4 (cells/μl) (IQR)	45 (15, 127.5)
Median CD4 (cells/μl) after treatment (IQR)	210.5 (142, 305)
Median duration of therapy before onset of lipoatrophy or lipodystrophy (years, IQR) (n=270)	2.63 (1.67, 4.00)
Median duration of therapy of nonlipoatrophy or nonlipodystrophy (years, IQR) (n=559)	5.53 (4.10, 7.18)

SD, standard deviation; IQR, interquartile range.

We first combined patients with LA and those with LD for analysis, as fat loss was commonly observed in both LA and LD cases. Among the 829 patients, 270 (32.6%) were diagnosed with LA or LD within the observation period. We kept on monitoring the other 559 (67.4%) until the end of the observation period and found that they developed neither LA nor LD (non-LA/LD cases). The median duration of therapy until diagnosis was 2.63 years for the LA or LD cases and 5.53 years for the non-LA/LD cases. In a follow-up of 4019.8 person-years, the incidence rate of LA or LD was 6.70 per 100 person-years. LA or LD cases were found more frequently in females (40.7%) than in males (26.6%), and the incidence rate per 100 person-years for females (9.11) was significantly higher than that for males (5.18) (IRR=1.76, 95% CI=1.37–2.25, $p<0.001$) (Table 2). Patients using d4T/3TC backbone regimens as starting drugs showed a significantly higher incidence rate per 100 person-years (7.71) than those with other non-d4T-containing (mostly AZT/3TC) regimens (3.49) (IRR=2.21, 95% CI=1.53–3.28, $p<0.001$) (Table 2). These findings were consistent with those of previous studies in other ethnic groups.[9,20,29,38,39] In contrast, age, transmission route, and CD4 counts pre-ART and at 1 year post-ART did not significantly affect the incidence of LA or LD (Table 2).

Fas −670AA, but not ApoC3 −455 or ARβ3 cod64 genotype, affects incidence of LA or LD in HIV-1-infected Thai patients

To elucidate the possible effects of genetic polymorphisms on the development of LA or LD, we determined the genotypes of *Fas* −670, *ApoC3* −455, and *ARβ3* cod64 of 829 HIV-1-infected Thai patients. LA or LD cases were found significantly more frequently among patients with the *Fas* −670AA genotype than in those with other *Fas* −670 genotypes (IRR=1.41, 95% CI=1.06–1.86, $p=0.015$, Table 2). The *Fas* −670AA genotype was found in 25.9% of the LA or LD cases and in 20.2% of the non-LA/LD cases during the observation period. Increased frequency of the *Fas* −670AA genotype in LA or LD cases was observed in both males and females (data not shown). In cases of *ApoC3* −455 and *ARβ3* cod64 genotypes, however, there were no statistically significant differences in the frequency of LA or LD between groups (Table 2). Adjustment for gender and starting drug regimens in multivariate Poisson regression analysis resulted in a slightly higher IRR for *Fas* −670AA (1.47, 95% CI=1.12-194, $p=0.005$, Table 2). This difference was statistically significant after correction for multiple testing of three SNPs ($p=0.015$).

Fas–670AA genotype affects incidence of LA but not that of LD in HIV-1-infected Thai patients

Among 270 LA and/or LD cases, 148 were diagnosed as LA singly and 105 were diagnosed as LD singly. There were 17 cases diagnosed as LA first who then subsequently developed fat accumulation. These 17 cases were grouped as LD cases. The median durations of therapy until diagnosis were 2.58 years for LA and 2.83 years for LD cases. When we analyzed LA and LD separately, females showed a higher IRR in both LA (1.60, 95% CI=1.14–2.24, $p=0.005$) and LD (2.31, 95% CI=1.59–3.38, $p<0.001$) (Tables 3 and 4)., Similarly, d4T-containing regimens showed significantly higher IRR in both

TABLE 2. UNIVARIATE AND MULTIVARIATE ANALYSIS OF ALL PATIENTS (N=829)

Factors	*Events*[1]	*Person-years*	*Events/100 person-years*	*Crude*		*Adjusted*[2] (n=*829*)	
				IRR (95% CI)	p-*value*	*IRR (95% CI)*	p-*value*
Gender							
Male	127	2449.9	5.18	1			
Female	143	1569.9	9.11	1.76 (1.37–2.25)	<0.001	1.70 (1.33–2.16)	<0.001
Transmission							
Unknown[3]	7	95.9	7.3	1			
Sexual	263	3924.0	6.7	0.92 (0.44–2.31)	0.782		
NRTIs							
No-d4T	33[4]	945.8	3.49	1			
d4T	237[5]	3074.0	7.71	2.21 (1.53–3.28)	<0.001	2.05 (1.42–2.96)	<0.001
Age×10 (years)				0.87 (0.74–1.02)	0.090		
CD4 at baseline[5]				0.9988 (0.9973–1.0003)	0.107		
CD4 after therapy				0.9497 (0.9987–1.0005)	0.458		
Fas−670							
AG/GG	200	3221.6	6.21	1			
AA	70	798.2	8.77	1.41 (1.06–1.86)	0.015	1.47 (1.12–1.94)	0.005
ApoC3−455							
CT/TT	206	3139.0	6.56	1			
CC	64	880.9	7.27	1.11 (0.82–1.47)	0.474		
ARβ3 cod64							
CC/CT	58	815.8	7.11	1			
TT	212	3204.0	6.62	0.93 (0.69–1.27)	0.621		

[1]Diagnosis of lipoatrophy or lipodystrophy.
[2]Gender, NRTIs, and *Fas*−670 were included as covariates.
[3]Including eight intravenous drug users.
[4]No-d4T group mainly used AZT. One case had received ddI instead of AZT as an initial regimen.
[5]CD4 cells/μl.
IRR, incidence rate ratios; NRTIs, nucleoside reverse transcriptase inhibitors; d4T, stavudine.

LA (2.46, 95% CI=1.48–4.36, $p<0.001$) and LD (2.10, 95%CI=1.23–3.80, $p=0.003$) (Tables 3 and 4). In the case of the *Fas* −670AA genotype, however, significantly higher IRR was observed only in LA (1.68, 95% CI=1.15–2.41, $p=0.006$) but not in LD (1.19, 95%CI=0.74–1.83, $p=0.433$) (Tables 3 and 4). The statistical significance of higher IRR of LA for *Fas* −670AA did not change after adjustment for gender and starting drug regimens in multivariate Poisson regression analysis (1.72, 95% CI=1.20–2.45, $p=0.003$, Table 3). The difference was statistically significant even after correction for multiple testing of three SNPs in two groups (LA or LD) ($p=0.018$). These results suggested that the *Fas* −670 genotype affects LA without fat accumulation.

Lipid profiles of HIV-1-infected Thai patients

We analyzed the serum lipid profiles of the patients. From the records of 829 patients, serum cholesterol levels were obtained in 610 and 628 cases at the first and last measurement of the observation period, respectively. Serum triglyceride (TG) levels were obtained in 646 and 716 cases at the first and last measurement, respectively. Serum HDL levels were obtained in 393 and 367 cases at the first and last measurement, respectively. Serum low-density lipoprotein (LDL) levels were obtained in 382 and 438 cases at the first and last measurement, respectively. Serum cholesterol levels showed a normal distribution, while serum TG, HDL, and LDL levels did not. Therefore, we used a nonparametric method to evaluate the statistical significance of differences in patient lipid profiles. Slight increases in serum cholesterol levels were observed in the LA (from median 194.5 to 206 mg/dl, $p=0.016$), LD (from median 194 to 205 mg/dl, $p=0.001$), and non-LA/LD cases (from median 195 to 200 mg/dl, $p=0.033$) during ART (Table 5). Similarly, significant increases in TG were observed in the LA (from median 130 to 183.5 mg/dl, $p=0.019$) and LD (from median 122 to 165.5 mg/dl, $p<0.001$), but not in non-LA/LD cases (from median 138 to 148.5 mg/dl, $p=0.359$; Table 5). Accordingly, the LA and LD cases showed significantly higher TG levels than the non-LA/LD cases at the last measurement ($p=0.007$; Table 5). These results indicated that lipid metabolism in LA and LD cases was more severely damaged by ART than that in non-LA/LD cases. It should also be noted here that the LA and LD cases showed weak but clear trends toward lower TG levels than non-LA/LD cases at the first measurement ($p=0.091$; Table 5). In fact, when combined, the LA and LD cases showed significantly lower TG levels (median 122 mg/dl) than the non-LA/LD cases (median 138 mg/dl) at the first measurement ($p=0.049$). On the other hand, HDL and LDL levels remained almost unchanged during the treatment period in the LA and LD, but non-LA/LD cases showed a slight decrease in LDL (from median 137 to 132.5 mg/dl, $p=0.026$; Table 5).

As the LA and LD cases showed similar lipid profiles, we combined the LA and LD cases again and analyzed the effects of patients' genotypes on the lipid profile together. The results indicated that the *ApoC3* −455CC genotype was associated with decreased serum cholesterol levels in non-LA/LD cases at the last measurement ($p=0.035$; Table 6). However, this association was not observed in LA and LD cases ($p=0.393$;

TABLE 3. LIPOATROPHY: RATE OF INCIDENCE AND MULTIVARIATE ANALYSIS (N=707)

Factors	Events[1]	Person-years	Events/100 person-years	Crude		Adjusted[2] (n=707)	
				IRR (95% CI)	p-value	IRR (95% CI)	p-value
Gender							
Male	76	2282.1	3.33	1			
Female	72	1350.9	5.33	1.60 (1.14–2.24)	0.005	1.56 (1.13–2.16)	0.007
Transmission							
Unknown	3	85.2	3.52	1			
Sexual	145	3547.8	4.09	1.16 (0.39–5.69)	0.865		
NRTIs							
No-d4T	17	880.5	1.93	1			
d4T	131	2752.5	4.76	2.46 (1.48–4.36)	<0.001	2.32 (1.40–3.85)	0.001
Age×10 (years)				0.88 (0.71–1.08)	0.228		
CD4 at baseline[3]				0.9985 (0.9964–1.0006)	0.151		
CD4 after therapy[3]				0.9991 (0.9979–1.0003)	0.151		
Fas−670							
AG/GG	105	2920.4	3.59	1			
AA	43	712.6	6.03	1.68 (1.15–2.41)	0.006	1.72 (1.20–2.45)	0.003
ApoC3−455							
CT/TT	118	2866.1	4.12	1			
CC	30	766.9	3.91	0.95 (0.61–1.43)	0.818		
ARβ3 cod64							
CC/CT	38	748.0	5.08	1			
TT	110	2885.0	3.81	0.75 (0.51–1.12)	0.134		

[1]Diagnosis of lipoatrophy.
[2]Gender, NRTIs, and *Fas*−670 were included as covariates.
[3]CD4 cells/μl.
IRR, incidence rate ratios; NRTIs, nucleoside reverse transcriptase inhibitors; d4T, stavudine.

TABLE 4. LIPODYSTROPHY: RATE OF INCIDENCE AND MULTIVARIATE ANALYSIS (N=681)

Factors	Events[1]	Person-years	Events/100 person-years	Crude		Adjusted[2] (n=681)	
				IRR (95% CI)	p-value	IRR (95% CI)	p-value
Gender							
Male	51	2234.3	2.28	1			
Female	71	1346.3	5.27	2.31 (1.59–3.38)	<0.001	2.19 (1.53–3.15)	<0.001
Transmission							
Unknown	4	86.0	4.65	1			
Sexual	118	3494.5	3.38	0.73 (0.28–2.71)	0.508		
NRTIs							
No-d4T	16	860.3	1.86	1			
d4T	106	2720.2	3.90	2.10 (1.23–3.80)	0.003	1.82 (1.07–3.09)	0.027
Age×10 (years)				0.85 (0.67–1.07)	0.171		
CD4 at baseline[3]				0.9988 (0.9966–1.0010)	0.278		
CD4 after therapy[3]				1.0000 (0.9990–1.0014)	0.696		
Fas−670							
AG/GG	95	2888.0	3.29	1			
AA	27	692.6	3.90	1.19 (0.74–1.83)	0.433	1.27 (0.83–1.95)	0.278
ApoC3−455							
CT/TT	88	2781.9	3.16	1			
CC	34	798.7	4.26	1.34 (0.88–2.02)	0.148		
ARβ3 cod64							
CC/CT	20	714.5	2.80	1			
TT	102	2866.1	3.56	1.27 (0.78–2.17)	0.328		

[1]Diagnosis of lipoatrophy.
[2]Gender, NRTIs, and *Fas*−670 were included as covariates.
[3]CD4 cells/μl.
Lipodystrophy cases included 17 cases diagnosed with both lipoatrophy and lipodystrophy.
IRR, incidence rate ratios; NRTIs, nucleoside reverse transcriptase inhibitors; d4T, stavudine.

TABLE 5. LIPID PROFILE OF PATIENTS

	LA cases[1]			*LD cases*[2]			*Non-LA/LD cases*[3]			
Lipid profile	n	*Median*	*IQR*	n	*Median*	*IQR*	n	*Median*	*IQR*	p-*value*[4]
Cholesterol (mg/dl)										
First test[5]	96	194.5	165.25–231.25	77	194	159.00–216.50	437	195	160.00–224.00	0.507
Last test[6]	121	206	177.50–236.00	95	205	180.00–239.00	412	200	176.500–227.75	0.106
p-value[7]		0.016			0.001			0.033		
TG (mg/dl)										
First test[5]	98	130	92.25–190.25	89	122	88.00–164.50	459	138.0	95.00–201.00	0.091
Last test[6]	140	183.5	109.50–285.00	116	165.5	103.75–256.25	460	148.5	97.00–224.75	0.007
p-value[7]		0.019			<0.001			0.359		
HDL (mg/dl)										
First test[5]	43	50	40.00–67.00	45	51	44.00–70.00	305	50	40.00–62.00	0.648
Last test[6]	63	52	42.00–66.00	51	52	42.00–63.00	253	50	40.00–62.00	0.419
p-value[7]		0.933			0.885			0.392		
LDL (mg/dl)										
First test[5]	41	136	117.00–174.00	51	133	116.00–168.00	290	137	108.00–167.00	0.731
Last test[6]	70	135.5	107.75–166.25	88	136.5	114.25–165.75	280	132.5	111.00–157.00	0.295
p-value[7]		0.777			0.735			0.026		

[1]Lipoatrophy cases.
[2]Lipodystrophy cases.
[3]Patients without lipoatrophy or lipodystrophy.
[4]Kruskal–Wallis test.
[5]The first measurement after patients started antiretroviral therapy.
[6]The last measurement of the observation period. For LA and LD cases, this corresponded to the diagnosis of LA and LD, respectively.
[7]Wilcoxon signed rank test.
LA, lipoatrophy; LD, lipodystrophy; IQR, interquartile range; TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Table 6) or at the first measurement in both LA and LD cases and non-LA/LD cases ($p=0.254$ and $p=0.583$, respectively). The *ApoC3* −455CC genotype was also found to be associated with elevated serum TG levels in the LA and LD cases at the time of LA or LD diagnosis ($p=0.021$; Table 6). In the non-LA/LD cases, the *ApoC3* −455CC genotype had virtually no effect on serum TG levels. These results suggested that the *ApoC3* −455CC genotype promotes dysregulation of lipid metabolism in LA or LD cases, even though this allele had no deleterious effect on the onset of LA or LD (Table 2). On the other hand, the *ApoC3* −455CC genotype affected neither HDL nor LDL serum levels (data not shown). The *ARβ3* cod64 genotype had no effect on the serum lipid data of any patient group (Table 6). Similarly, *Fas* −670AA, which was found to be significantly associated with LA, did not show any effects on serum lipid levels (Table 6).

Discussion

In the present study, we evaluated the influence of SNPs involved in apoptosis and lipid metabolism on developing LA or LD in Thai HIV-1 patients. Specifically, we analyzed the SNPs of the *Fas* A–670G, *ApoC3* T–455C, and *ARβ3* Tcod64C in patients undergoing ART.

Of the SNPs investigated, our findings indicated that the AA genotype of *Fas* −670 was significantly associated with the risk of LA but not that of LD. This result is consistent with that of a previous study in Italian HIV patients by Zanone Poma *et al.*[29] It was suggested that adipocyte apoptosis induced by mitochondrial toxicity of NRTIs was the primary mechanism involved in LA.[15] Consistent with this suggestion, increased *Fas* gene expression levels were observed in adipose tissue of HIV-1 patients with NRTI-associated LA.[40] The *Fas* A–670G polymorphism is located in the γ-interferon activation site, to which transcription factors such as signal transducer and activator of transcription (STAT) bind.[41] Therefore, this SNP may affect the level of *Fas* gene transcription and may induce apoptosis, consequently increasing the risk of LA. The *Fas* A–670G polymorphism has been reported to be associated with several autoimmune diseases and inflammatory disorders,[41–43] which may involve a mechanism similar to LA. The frequency of the AA genotype of *Fas* −670 in 829 HIV-1-infected Thais was 22.1%, which is similar to the percentages in Europeans and Japanese but is markedly higher than that in Africans. As *Fas* −670AA caused a 70% increase in risk of LA, this genotype is a candidate for future pretherapy genetic screening, although it is still necessary to find other SNPs that increase the risk of LA for accurate screening. It is also necessary to confirm our findings on *Fas* −670AA in a larger patient cohort.

On the other hand, the frequency distribution of the *ARβ3* cod64 and *ApoC3* −455 genotypes was not significantly different between LA or LD cases and non-LA/LD cases. The *ARβ3* Tcod64C polymorphism failed to show any effect on lipid metabolism among Thai patients in this study. The results for *ARβ3* cod64 were consistent with a previous report that showed no association of this polymorphism with lipid metabolism during weight loss in obese subjects.[44] However, in the Italian cohort, this genotype was associated with the risk of developing LA.[29] Several studies have reported that people with this SNP showed an increased risk of developing obesity and glucose intolerance,[32,33] whereas other studies failed to find any correlation between this mutation and body mass index.[45,46]

The results for *ApoC3* −455 were also not consistent with those of the Italian cohort, which showed an association of the

TABLE 6. PATIENT GENOTYPE AND LIPID PROFILE

	Cholesterol (mg/dl)											
	LA and LD cases[1]						*Non-LA/LD cases*[2]					
	First test			*Last test*			*First test*			*Last test*		
Genotype	n	*Median*	*IQR*	n	*Median*	*IQR*	n	*Median*	*IQR*	n	*Median*	*IQR*
ApoC3−455 CC	42	202	172.50–225.00	51	214	183.00–239.00	86	196	154.75–220.25	86	193.5	164.50–220.50
ApoC3−455 CT/TT	131	191	159.00–218.00	165	204	179.50–240.00	351	195	161.00–224.00	326	202.5	177.75–229.00
p-value[3]		0.254			0.393			0.583			0.035	
ARβ3 cod64 TT	138	194	159.75–224.25	169	206	177.50–216.50	347	198	162.00–224.00	332	202	175.25–228.00
ARβ3 cod64 CT/CC	35	194	177.00–207.00	47	202	178.00–246.00	90	189	156.00–223.50	80	194	174.25–221.00
p-value[3]		0.806			0.658			0.360			0.328	
Fas−670 AA	39	192	158.00–217.00	53	211	182.50–240.00	87	197	158.00–223.00	85	201	176.50–232.50
Fas−670 AG/GG	134	195	165.75–223.50	163	205	173.50–236.75	350	195	161.00–224.00	327	199	175.50–226.00
p-value[3]		0.259			0.602			0.820			0.493	
	TG (mg/dL)											
	LA and LD cases[1]						*Non-LA/LD cases*[2]					
	First test			*Last test*			*First test*			*Last test*		
Genotype	n	*Median*	*IQR*	n	*Median*	*IQR*	n	*Median*	*IQR*	n	*Median*	*IQR*
ApoC3−455 CC	44	132	102.25–209.75	61	222	129.00–327.50	91	145	99.00–204.00	94	158	99.25–210.25
ApoC3−455 CT/TT	143	122	87.00–179.00	195	166	101.00–248.25	368	136	93.25–201.00	366	144	97.00–226.00
p-value[3]		0.277			0.021			0.771			0.945	
ARβ3 cod64 TT	150	126	93.00–185.75	198	175	77.50–281.00	363	140	97.00–199.00	360	144.5	93.25–218.75
ARβ3 cod64 CT/CC	37	108	82.50–160.00	58	167.50	109.00–274.50	93	124.5	77.75–206.75	100	154	107.00–232.50
p-value[3]		0.174			0.973			0.368			0.500	
Fas−670 AA	42	120	89.50–162.75	64	167	99.25–270.75	92	128	94.00–202.50	96	123.5	96.50–223.75
Fas−670 AG/GG	145	123	89.00–184.00	192	175	103.00–260.00	367	140	95.00–201.00	364	152	97.00–224.75
p-value[3]		0.580			0.530			0.655			0.498	

[1]Lipoatrophy and lipodystrophy cases.
[2]Patients without lipoatrophy or lipodystrophy.
[3]Kruskal–Wallis test.
LA, lipoatrophy; LD, lipodystrophy; IQR, interquartile range.

ApoC3 −455T allele with LA. However, it was also reported that the *ApoC3* −455C but not the *ApoC3* −455T allele was associated with metabolic syndrome.[47] In fact, a recent study in French HIV patients showed that the *ApoC3* −455C but not the *ApoC3* −455T allele is associated with the severity of ART-induced dyslipidemia and occurrence of LD.[48,49] Therefore, the precise role of the *ApoC3* T–455C polymorphism in LA or LD during ART should be carefully reevaluated in a larger cohort of HIV patients.

Nevertheless, our study also showed that the *ApoC3* −455CC genotype was associated with elevated serum levels of TG, which was consistent with the findings of a previous report showing that the *ApoC3* −455C allele was associated with metabolic syndrome.[47] These data suggest that the mechanism underlying the promotion of LA and LD in HIV-infected individuals receiving ART may be different from that promoting metabolic syndrome. However, the differences in results may be due to ethnic differences in the cohorts. It was reported that genetic polymorphisms in hepatic lipase showed different effects on plasma lipid levels in different ethnic groups.[50] Foulkes *et al.* found race- and ethnic-specific differences in plasma lipid levels under ART, as well as differences in the influence of *ApoC3* T–455C and the other SNP in *ApoC3* gene on the development of protease inhibitor-related hypertriglyceridemia.[51] Further studies including reevaluation of these SNPs in larger patient cohorts are therefore necessary to elucidate the precise mechanisms underlying LA and LD in HIV-infected individuals receiving ART.

In conclusion, the *Fas* −670AA genotype, but not the *ApoC3* −455 or *ARβ3* cod64 genotypes, affected the incidence of LA in HIV-1-infected Thai patients. None of these alleles affected the incidence of LD. On the other hand, the *ApoC3* −455CC, but not the *ARβ3* cod64 genotype, affected the serum levels of TG.

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Author Disclosure Statement

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Elicitation of Both Anti HIV-1 Env Humoral and Cellular Immunities by Replicating Vaccinia Prime Sendai Virus Boost Regimen and Boosting by CD40Lm

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Abstract

For protection from HIV-1 infection, a vaccine should elicit both humoral and cell-mediated immune responses. A novel vaccine regimen and adjuvant that induce high levels of HIV-1 Env-specific T cell and antibody (Ab) responses was developed in this study. The prime-boost regimen that used combinations of replication-competent vaccinia LC16m8Δ (m8Δ) and Sendai virus (SeV) vectors expressing HIV-1 Env efficiently produced both Env-specific $CD8^+$ T cells and anti-Env antibodies, including neutralizing antibodies (nAbs). These results sharply contrast with vaccine regimens that prime with an Env expressing plasmid and boost with the m8Δ or SeV vector that mainly elicited cellular immunities. Moreover, co-priming with combinations of m8Δs expressing Env or a membrane-bound human CD40 ligand mutant (CD40Lm) enhanced Env-specific $CD8^+$ T cell production, but not anti-Env antibody production. In contrast, priming with an m8Δ that coexpresses CD40Lm and Env elicited more anti-Env Abs with higher avidity, but did not promote T cell responses. These results suggest that the m8Δ prime/SeV boost regimen in conjunction with CD40Lm expression could be used as an immunization platform for driving both potent cellular and humoral immunities against pathogens such as HIV-1.

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Introduction

An effective HIV vaccine should elicit both antibodies [1] and cell-mediated immune responses in order to control HIV infection. Since the majority of clinical isolates of human immunodeficiency virus type 1 (HIV-1) are highly resistant to neutralizing antibodies and antigenically variable, major efforts have been aimed at eliciting cellular immunity against less variable antigens. Typical prime/boost strategies using DNA and replication-defective viral vectors have been extensively examined. These regimens efficiently elicit cellular responses including cytotoxic T cells (CTL), but are less effective at eliciting humoral responses. For example, adenovirus and vaccinia virus-based vectors expressing Gag, Nef, and other components of HIV-1 have been shown, in nonhuman primates [2–5] and in human trials [6,7], to elicit considerable multifunctional T cell responses and control early viral replication to some extent. These preparations, however, did not induce a sufficient level of immunity to protect vaccinees from HIV/simian immunodeficiency virus (SIV) infection in the absence of neutralizing antibodies [8]. Therefore, more potent immunogens and better vaccination regimens are required.

The RV144 trial that included priming with a recombinant canarypox vector, ALVAC-HIV vCP1521, followed by booster with the HIV-1 envelope gp120 protein, AIDSVAX gp120 clades B and E, plus an alum adjuvant showed a modest level of efficacy in reducing HIV-1 infection rates in Thailand [9]. Extended analysis of this HIV vaccine trial showed that it is the vaccine trial to succeed in eliciting IgG antibodies to the V1V2 region of Env, and the presence of these antibodies were inversely correlated to the rate of infection [10], suggesting an importance to elicit anti HIV-1 specific antibodies. Accordingly, both antibodies and cell-mediated immune responses should be considered for the vaccine development in order to control HIV infection.

Replication-competent vaccinia virus (VV) that has been proven to be safe in human vaccination against smallpox may be a good vehicle candidate. Among several vaccinia strains, LC16m8 has an extremely low neurovirulence profile, comparable to the replication incompetent vaccinia viruses MVA and NYVAC, and is safe in immune compromised animals [11–13]. LC16m8 is able to induce immunity at levels similar to the original Lister (LO) strain and the US licensed vaccine dryvax strain [11–13], and no serious adverse effects were detected in the administration of LC16m8 to

100,000 infants and 3,000 adults [14]. However, LC16m8 is genetically unstable and can spontaneously generate more virulent revertants. To improve the safety of LC16m8, we identified the B5R gene responsible for the reversions and constructed the genetically stable LC16m8Δ (m8Δ), which is essentially the same as LC16m8 in antigenicity, safe in mice and rabbits, and much more immunogenic than the MVA strain [13]. Thus, m8Δ may be a better vehicle for vaccines. Indeed, immunization in a prime-boost strategy using DNA and m8Δ expressing SIV Gag elicited 7–30 fold more IFN-γ producing T cells in mice than were produced using the non-replicating vaccinia DIs strain [15].

The Sendai virus (SeV) is a non-segmented negative-strand RNA virus belonging to the paramyxovirus family and is considered nonpathogenic in humans [16–19]. A SeV vector expressing the SIV gag gene elicits SIV-specific CTL very efficiently and controlled SIV replication in a subset of immunized macaques [20,21]. Thus, the SeV vector may be another candidate for a better immunogen.

In addition to adopting better vaccination vehicles, combining these with an immune stimulating factor could produce a better efficacy. The CD40 ligand (CD40L, CD154), which belongs to the tumor necrosis factor (TNF) family, is a 39 kDa type II membrane glycoprotein that is predominantly expressed on activated $CD4^+$ T cells [22]. CD40, the TNF receptor superfamily member that is the CD40L receptor, is expressed on all antigen-presenting cells (APCs), including macrophages, dendritic cells (DCs) and B lymphocytes [23]. Interactions between these receptors and ligand play a central role in adaptive immune responses including maturation of DCs and class switching of immunoglobulin genes [24]. Coexpression of CD40L with immunogens has the potential to enhance both humoral and cellular immune responses in various regimens [25–29]. However, one concern is that high levels of CD40L, mainly resulting from cleavage to produce a soluble form, may have deleterious side effects and could lead to systemic inflammatory responses and cardiovascular disease. A non-cleavable CD40L, CD40Lm, which was constructed with point mutations in the membrane proximal region, was reported to be less toxic in vivo [30]. Therefore, coexpression of CD40Lm may further enhance the induction of immune responses to HIV-1 without adverse effect.

To identify an improved vaccination regimen that elicits higher levels of anti-HIV-1 humoral and cellular responses, various combinations of vaccine preparations were tested in this study using the vaccinia virus m8Δ and SeV vectors expressing HIV-1 Env in conjunction with the coexpression of human CD40Lm (hCD40Lm).

Materials and Methods

Ethics Statement

All animal experiments were conducted according to the *Guide for the Care and Use of Laboratory Animals*, Institute for Genetic Medicine, Hokkaido University. Study approval was issued by the Animal Care Committees of Hokkaido University.

Construction of HIV-1 envelope expression plasmids

The region encoding the Rev and Env genes of the HIV-1 $_{JR\text{-}CSF}$ genome (5981–8782 nt) was inserted into the EcoR1 restriction site of the mammalian expression vector pCAGGS [31,32] to generate pCAGGS-JRCSFrev/env. To confirm the expression of gp160, a sequence-verified pCAGGS-JRCSFrev/env was transfected into 293T cells using polyethylenimine (Polysciences, Warrington, US) [33]. Forty-eight hours after transfection, 293T cell lysates were collected and proteins were fractioned on 10% SDS polyacrylamide gels and transferred to a nitrocellulose filter (Schleicher & Schuell). Immunoblot analysis was performed with HIV-1-infected human antiserum and alkaline phosphatase-conjugated anti-human IgG (Promega, Sunnyvale, US), and then visualized using NBT/BCIP.

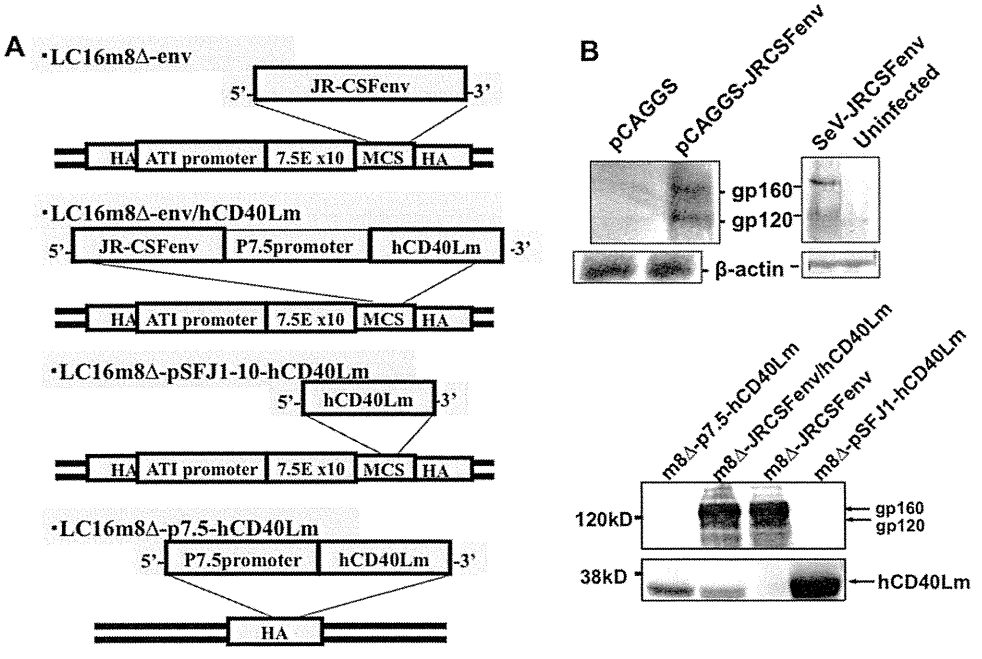

Figure 1. Structures and expression of recombinant DNA and LC16m8Δ. (A) Structures of four different recombinant m8Δs. (B) Profiles of Western blotting for Env and human CD40Lm expressed by infection with the HIV-1 Env expression plasmid, pCAGGS-JRCSFrev/env, SeV-env, and recombinant m8Δs are presented.
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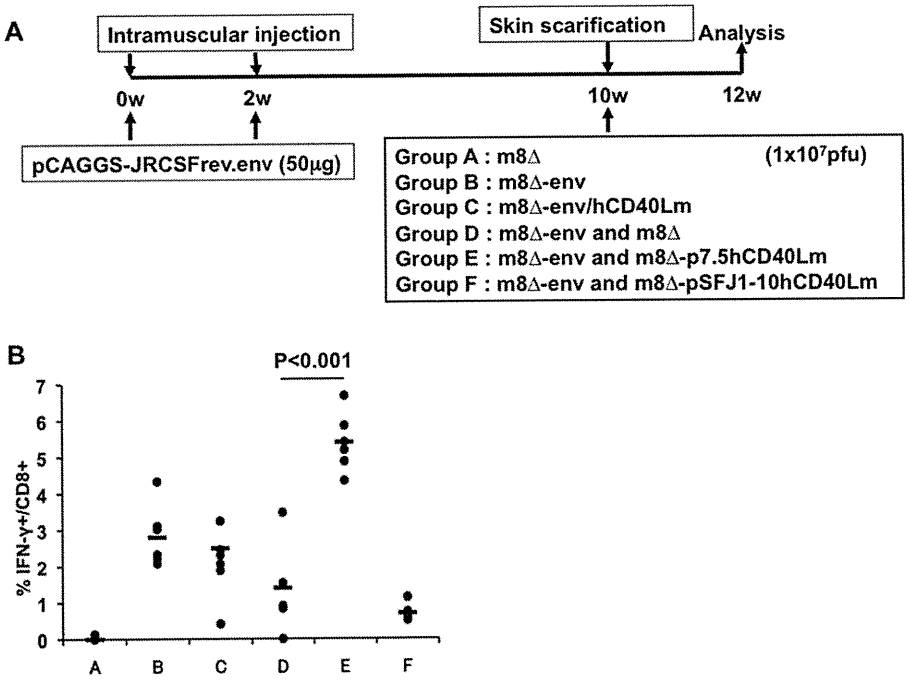

Figure 2. Cellular immunity elicited by a regimen consisting of DNA priming followed by vaccinia m8Δ boosts. (A) Schematic schedules of DNA prime/m8Δ-Env boost vaccination protocol. Mice were immunized twice with pCAGGS-JRCSFrev/env and boosted with various vaccinia viruses as follows. Group A: m8Δ; group B: m8Δ-Env; group C: m8Δ-Env/hCD40Lm; group D: m8Δ-Env plus m8Δ; group E: m8Δ-Env plus m8Δ-p7.5hCD40Lm; and group F: m8Δ-Env plus m8Δ-pSFJ1-10hCD40Lm. (B) Comparison of Env peptide-specific $CD8^+$ T cell responses. The frequencies of IFN-γ^+ $CD8^+$ T cells in gated $CD8^+$ T cell compartment was determined by intracellular cytokine staining (ICS) and FACScalibur/FACScanto analysis.
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Construction of recombinant LC16m8Δ

The four strains of recombinant m8Δ used in this study are constructed as follows. To construct a m8Δ that expresses the full HIV-1 $_{JR\text{-}CSF}$ Env gene under the control of the vaccinia virus promoter in pSFJ1-10 [21], the AvrII-XhoI fragment of the JR-CSF genome was subcloned into the pJW322 plasmid [34] that had been digested with AvrII and SalI, and the vaccinia virus transcription termination signals (TTTTTNT) in the envelope gene were synonymously mutated using an in vitro mutagenesis kit (Stratagene). The coding region for gp160 was then amplified by PCR using the forward primer TTTCGGACCGCCACCATGAGAGTGAAGGGGATCAGG (underline shows the RsrII site) and the reverse primer ATAGGCCGGCCTTATAGCAAAGCCCTTTCCAAGC (underline shows the FseI site). The PCR product was ligated into the LC16m8Δvnc110 [15] genome that had been digested with FseI and RsrII. The ligated DNA was transfected into BHK cells that were infected with

Table 1. Mice immunization protocol used in this study.

Vaccination group	Prime	Boost
A	pCAGGS-JRCSF rev.env	M8Δ
B	pCAGGS-JRCSF rev.env	M8Δ-env
C	pCAGGS-JRCSF rev.env	M8Δ-env/hCD40Lm
D	pCAGGS-JRCSF rev.env	M8Δ-env+ m8Δ
E	pCAGGS-JRCSF rev.env	M8Δ-env + m8Δ-p7.5hCD40Lm
F	pCAGGS-JRCSF rev.env	M8Δ-env + m8Δ-pSFJ1-10hCD40Lm
G	M8Δ-env + m8Δ-p7.5hCD40Lm	SeV-JRCSF env
H	M8Δ-env + m8Δ	SeV-JRCSF env
I	M8Δ-env/hCD40Lm	SeV-JRCSF env
J	M8Δ-env	SeV-JRCSF env
K	M8Δ	SeV-JRCSF env
L	Naive	SeV-JRCSF env
M	pCAGGS-JRCSF rev.env	SeV-JRCSF env

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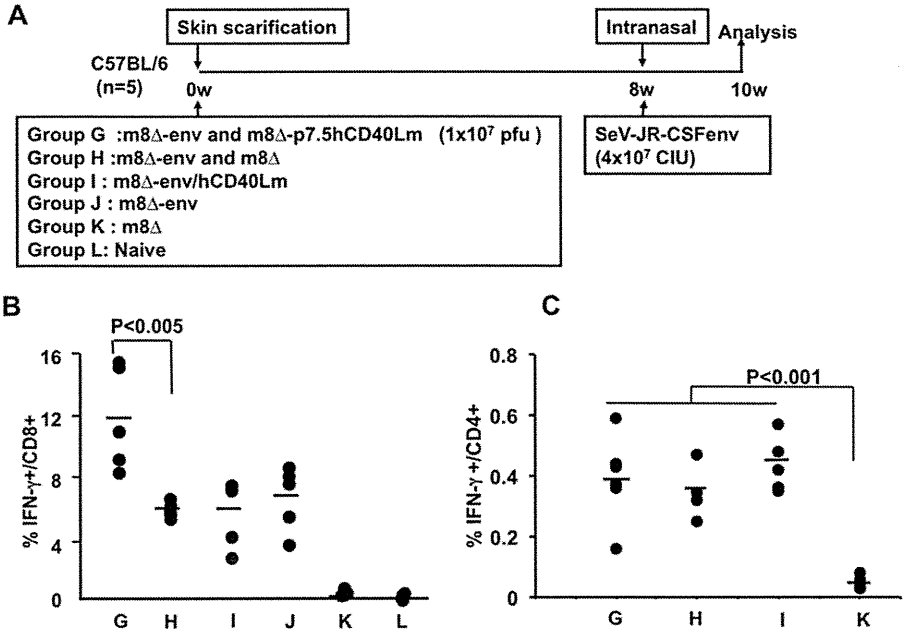

Figure 3. Immunity elicited by Env and CD40Lm expressing m8Δ prime followed by recombinant SeV boost. (A) Schematic schedules of m8Δ prime/rSeV-Env boost vaccination protocol. Mice were primed with various vaccinia viruses followed by Env expressing SeV boost. Group G: primed with m8Δ-Env plus m8Δ-p7.5hCD40Lm; group H: m8Δ-Env plus m8Δ; group I: m8Δ-Env/hCD40Lm; group J: m8Δ-Env; group K: m8Δ; group L: naive. (B) Comparison of Env peptide-specific $CD8^+$ T cell responses. The percentage of IFN-γ^+ expressing cells in gated $CD8^+$ T cell compartment was measured by ICS and FACS analysis. (C) The percentage of IFN-γ^+ T cells in gated $CD4^+$ T cell compartment.
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canarypox virus, as described previously [14]. The vaccinia virus constructed was designated LC16m8Δ-env. For the m8Δ-p7.5hCD40Lm construct, the human CD40Lm gene [35] was inserted into pVR1 containing the p7.5 vaccinia virus promoter [36] at the BamHI and AvaI sites, which interrupts the HA gene sequence [37,38]. The construct was transfected into m8Δ-infected BHK cells, followed by selection based on an HA^- phenotype, as described previously [38,39]. For the m8Δ-pSFJ1-10hCD40Lm construct, the hCD40Lm gene was inserted into the XmaI and Not I sites of pBHAR that contains the pSFJ1-10 sequence inserted within the vaccinia virus HA gene [40]. For the m8Δ-Env/hCD40Lm construct that coexpresses pSFJ1-10-driven Env and p7.5-driven hCD40Lm, the Env gene fragment with EcoRI and SacI sites, and the p7.5 promoter-hCD40Lm fragment with SacI and XmaI sites were generated by PCR and the construct was inserted into the EcoRI and XmaI sites of pBR322. Then, the entire env-p7.5 promoter-hCD40Lm region was amplified by PCR and ligated into the m8Δvnc110 genome, as above. A schematic diagram of each m8Δ construct is shown in Fig. 1A. To verify protein expression, RK13 cells were infected with these recombinant m8Δs at a moi of 5 and cultured for 24 h at 33°C. Cell lysates were prepared, and analyzed by immunoblot analysis using the HIV-1-infected human antiserum or mouse anti-hCD40Lm mAb as the primary antibodies.

Construction of SeV expressing the JR-CSF env gene

Potential EIS sequences that may affect transcription of SeV were identified in the JR-CSF Env gene, and nucleotide substitutions that did not alter the amino acid sequence were made using in vitro mutagenesis with the following primers: mutation 1 forward primer, CCATCGTCTTCACTCACTCCT-CAGGAGGGGATCCAGAAATTG; mutation 1 reverse primer, GAATAACACTTT AAAACAGATAGTTGAGAAGCTCCGC GAGCAGTTCAACAACAAGACCATCGTCTTCACTCACT CCTCAGGAG; mutation 2 forward primer, GTGAAGATC GAACCATTAGGAGTAGCACCCACCAAGGCAAAG; mutation 2 reverse primer, GAGACATGAGGGACAATTGGA GAAGTGAGCTCTACAAGTACAAGGTCGTGAAGATCGA ACCATTAGGAGTA; mutation 3 forward primer, CGCA TCGTG TTCTCTGTACTTTCTATAGTGAATAGAGTTAG GCAGG; and mutation 3 reverse primer, GTTTGACATAA CAAAATGGCTGTGGTACATCAAGATCTTCATCATGA T CGTGGGAGGCCTGATCGGTCTCCGCATCGTGTTCTC TGTACTTTCTATAG. The mutated env fragment was subcloned into pSeV/ΔF and replication incompetent SeVJRCSFenv recombinant virus was constructed as previously described [41].

Immunization of mice

Seven-week-old female C57BL/6J mice (CLEA Japan, Tokyo) were primed twice at 2 week intervals by intramuscular (i.m.) injection with 100 μl PBS containing 50 μg pCAGGS-JRCSFrev/env. Eight weeks later, animals were boosted with 1×10^7 PFU of the recombinant m8Δ by skin scarification (s.s.) [42]. Alternatively, mice were primed with 1×10^7 PFU of the recombinant m8Δ by s.s. and eight weeks later boosted with 4×10^7 CIU of recombinant SeV intranasally (i.n.). Two weeks after the last immunization, vaginal fluids were collected, and mice were then sacrificed to collect sera and spleens.

Intracellular cytokine staining (ICS)

Splenocytes were stimulated with 10 μg/ml HIV-1 consensus subtype B Env (15-mer) peptides (AIDS Research and Reference Reagent Program) in the presence of Alexa Fluor-488 labeled anti-mouse CD107a (2.5 μg/ml) and brefeldin A (BD Biosciences) for 6 h. The cells were then washed and stained with PE-labeled anti-mouse CD8 (eBioscience, San Diego, US) and Pacific Blue-labeled anti-CD4 (eBiosience, San Diego, US) mAbs for 30 min at 4°C.

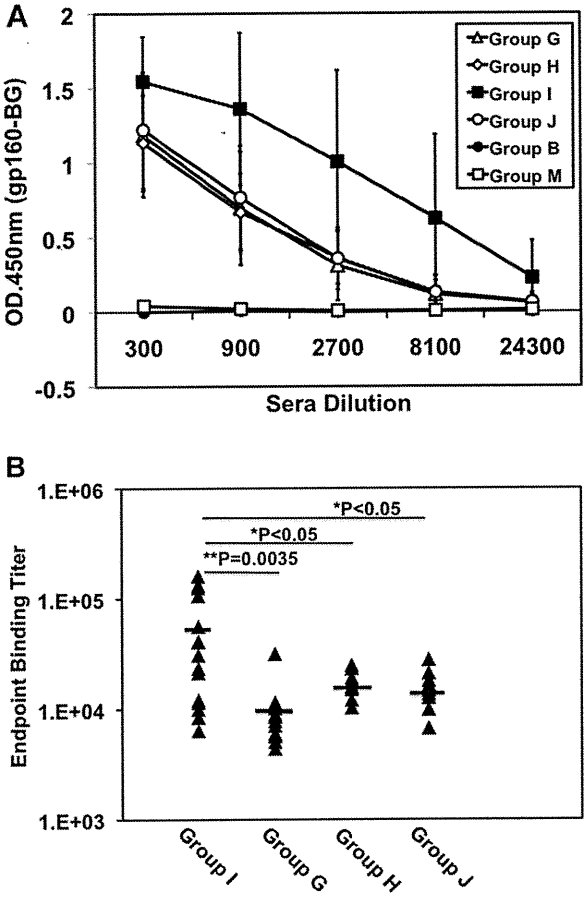

Figure 4. Priming with the coexpression vector m8Δ-Env/hCD40Lm induces greater amounts of Env-specific antibodies. Serum from individual immunized mice was analyzed using an HIV-1_{JR-CSF} gp160 ELISA assay. The plates were developed with HRP-conjugated anti-mouse IgG antibody. (A) The titer of Env-specific antibodies was determined by OD_{450} values subtracted from the background values. Data are mean ± SD of the Env-specific antibody titer of all animals in each group (n = 16 for group G and I; n = 8 for group H and J; n = 5 for group B and M). (B) Endpoint binding titer of sera against HIV-1 JR-CSF gp160 from each of vaccine groups was plotted. The titer in the group primed with m8ΔEnv/hCD40Lm was significantly different from all other groups. There are no significant differences in env-specific antibody titer among the group G, H and J.
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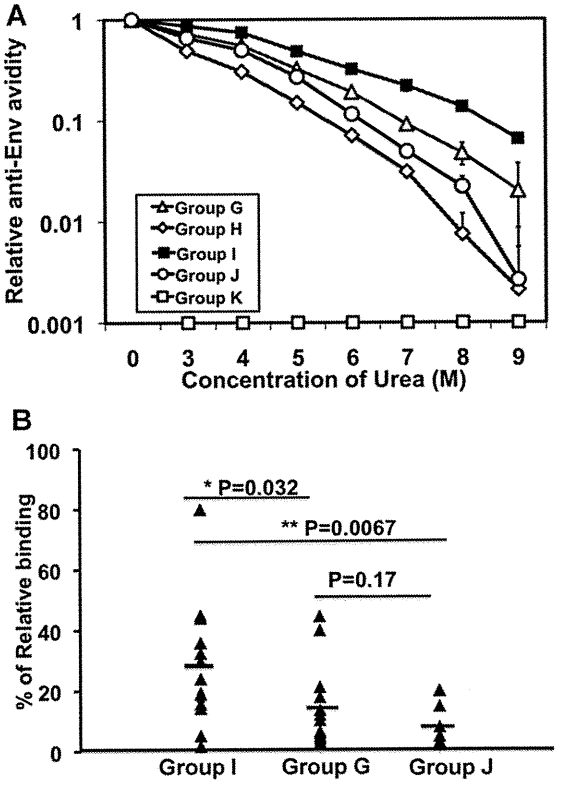

Figure 5. Priming with m8Δ-Env/hCD40Lm elicits Env-specific antibodies with higher avidity. The avidity of serum antibodies for HIV-1 Env was determined based on the resistance of antibody-gp160 binding to disruption by treatment with urea. (A) Pooled serum from immunized mice of each group was applied to a HIV-1_{JR-CSF} gp160-coated ELISA plate at a 1:300 dilution, as described above. After antibody-antigen binding, the plates were treated with increasing concentrations of urea (3–9 M) for 30 min at room temperature, followed by the ELISA procedure. (B) Dissociation analysis of 300 fold-diluted serum from individual mice using 7 M urea. The percentage of antibody remaining after urea treatment was plotted, and the mean and P values were calculated.
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After washing, the cells were permeabilized with Cytofix/Cytoperm solution (BD Bioscience, Franklin lakes, US) and stained with APC-labeled anti-mouse IFN-γ (eBiosience, San Diego, US) mAb. Then, the samples were subjected to analysis using a FACScalibur or FACScantoII instrument (BD Bioscience, Franklin lakes, US). Data were analyzed with the FlowJo software (Tree Star). The frequencies of IFN-γ+ or IFN-γ, CD107a double positive T cells among CD4 or CD8 gated lymphocytes were determined.

Purification of IgG from mouse serum

Twenty micro liter of mice sera randomly selected from group G, I, J and control were mixed with 20 μl of protein A Sepharose 4 Fast Flow resins (GE healthcare life science, Tokyo, Japan) that was pre-washed with TBS buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl), and incubate at 4°C for 1 hr with rotation. The Protein A sehparose resins were then rinsed 3 times with TBS buffer. The antibodies were eluted with 50 μl of elution buffer (50 mM glycine-HCl, pH 2.5) at 4°C for 5 min followed by addition of 2.5 μl of 1 M Tris-HCl (pH 9.0) to adjust pH to 7. The isotype of the purified antibodies were analyzed with BD cytometric bead array system according to the manufacturer's instruction.

Serum antibody measurements by enzyme-linked immunosorbent assay (ELISA)

To prepare ELISA plates coated with HIV-1_{JR-CSF} gp160, 293T cells (2.5×10^6 cells) that had been transfected with 10 μg of pCAGGS-JRCSFrev/env 2 days before were lysed in 1 ml of TMN buffer (10 mM Tris-HCl, pH 7.4, 1.5 mM $MgCl_2$, 140 mM NaCl, 0.5% NP40 and 1X complete protease inhibitor cocktail (Roche Applied Science, Sandhofer Strasse, Germany)) and desalted by ultrafiltration using a Centricon YM-100 (Millipore). After dilution to a final concentration of 3.0 μg/ml protein with coating buffer (eBioscience, San Diego, US), the cell lysates were added to Maxisoap 96 well ELISA plates (NUNC) at 100 μl aliquots/well and incubated overnight at 4°C. The wells were washed twice with PBS plus 0.05% Tween 20 (PBS-T) and blocked with PBS-T containing 5% skim milk for 1 h at room temperature. Subsequently, 100 μl samples of sera, serially diluted with the blocking solution, were added to the wells and incubated for 2 h at room temperature. Plates were washed five times with

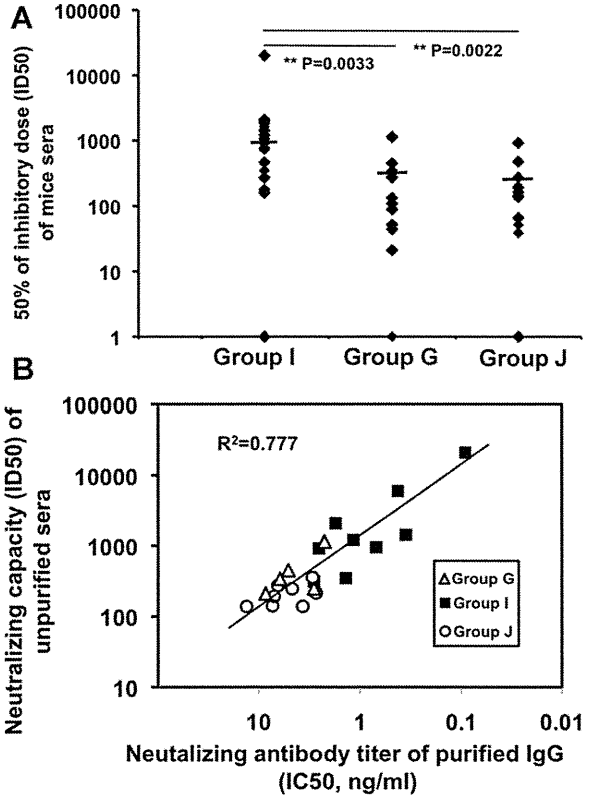

Figure 6. Neutralizing antibodies elicited in the Env expressing LC16m8Δ prime/SeV boost regimen. (A) ID_{50} of individual mouse serum against SF162 envelope-pseudotyped virus was determined using TZM-bl cells. Groups I, G and J consisted of 21, 17 and 14 mice, respectively. (B) Antibodies from randomly selected mice sera from group G, I, J and control were purified using protein A Sepharose. ID_{50} of individual mouse serum against SF162 envelope-pseudotyped virus vs 50% inhibitory concentration (IC_{50}) (concentration of purified IgG that caused a 50% reduction in the RLU compared to virus control) of individual purified IgG was plotted. The regression line and R square are shown.
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PBS-T. Aliquots (100 µl) of horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Promega, Sunnyvale, US), diluted 1:2500 from the stock solution, were added and incubated for 1 h at room temperature. The plates were washed five times with PBS-T and TMB ELISA substrate solution (eBioscience, San Diego, US) was added. After a 15 min incubation at room temperature, the reaction was stopped by addition of 1 M H_3PO_4. Optical density at 450 nm (OD_{450}) was measured using a plate reader (PerkinElmer). To calculate the amounts of Env-specific antibodies, OD values were background subtracted using the OD_{450} for wells coated with cell lysates prepared from pCAGGS-transfected 293T cells.

To assess the avidity of anti-Env antibodies, 300 fold-diluted sera were added to the wells of ELISA plates and incubated as above. The plates were then washed with 3–9 M urea and subjected to the above procedure for quantification of anti-Env antibodies. Relative avidity was estimated as the ratio of absorbance after and before the urea wash. The concentration of urea required to release 50% of the bound antibody (half-maximal effective dose (ED_{50})) was calculated by linear regression analysis of plots of the probit values vs. urea concentration.

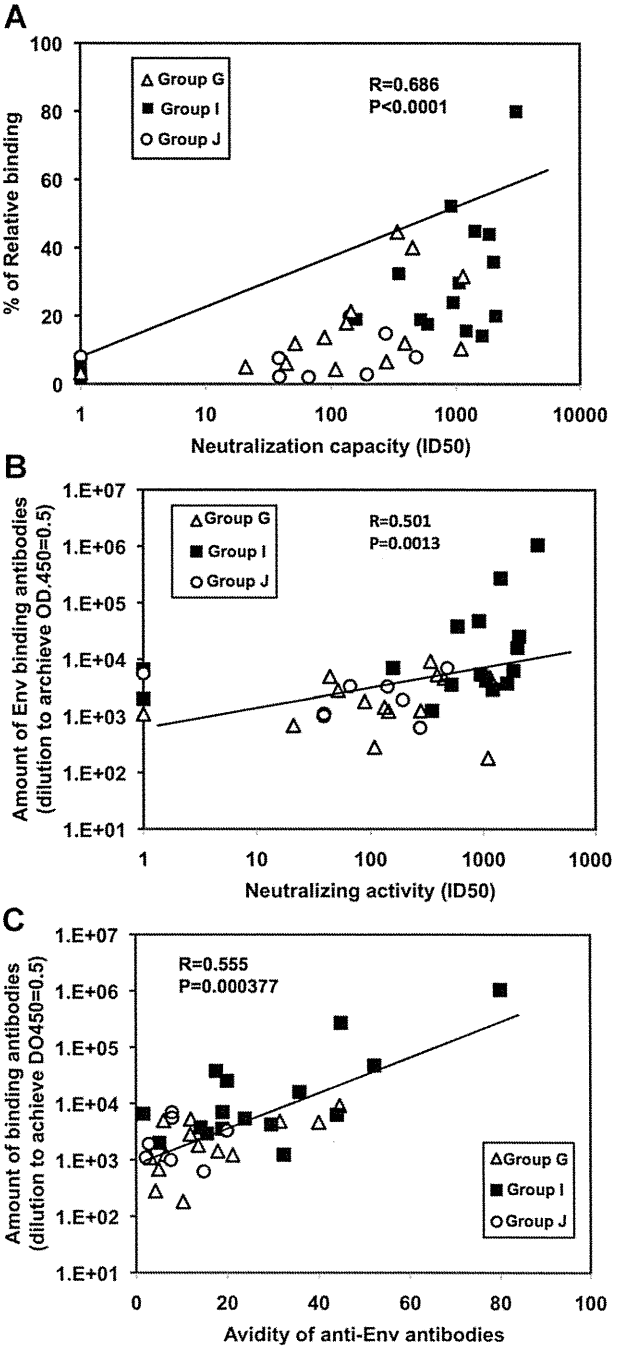

Figure 7. Correlation between neutralization activity against HIV-1 SF162 and the amount and avidity of anti-Env antibodies. The trendline and R and P values are displayed. (A) Correlation between neutralization activity against HIV-1 SF162 and the avidity of Env antibodies. (B) Correlation between the amount of anti-Env antibodies and neutralization activity. (C) Correlation between the amount of anti-Env antibodies and their avidity.
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Neutralizing antibody measurements

The titer of neutralizing Abs (nAbs) was assessed based on the reduction of luciferase reporter gene expression in TZM-bl cells after a single round of Env-pseudotyped virus infection, as described previously [35,43]. HIV-1 Env-pseudotyped virus (1000 TCID50/ml) was incubated with five-fold serially-diluted test sera in triplicate. One set of control wells received the cells plus

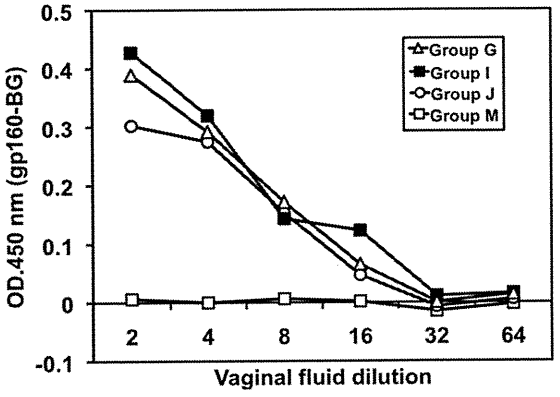

Figure 8. Env-specific mucosal IgG elicited by the regimen of Env expressing LC16m8Δ prime/SeV boost. Vaginal fluid pooled from 5–6 immunized mice was analyzed by HIV-1_{JR-CSF} gp160 ELISA assay. The ELISA was performed as indicated in Fig. 4. The data shown are representative results of three independent experiments.
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virus (virus control), another set of wells received cells only (background control). To examine the non-specific effect of sera, MuLV Env or VSV glycoprotein-coated pseudotype virus was included. The 50% inhibitory dose (ID_{50}) was calculated as the serum dilution that caused a 50% reduction in the relative light unit (RLU) values compared to virus control wells after subtraction of background RLU.

Measurement of IgA and IgG in vaginal fluid

To collect vaginal samples, a total of 400 μl of PBS was flushed into the vagina and collected into a microcentrifuge tube. The mucosal washings were centrifuged at 1000 g for 10 min. The supernatants were collected and stored at −20°C until assay. Total IgA or IgG concentration in the vaginal fluid was quantified using a mouse IgA/IgG ELISA quantitation kit (Bethy Laboratories, Montgomery, US) according to the manufacturer's instructions. The amount of HIV-1 gp160-specific IgA or IgG was determined using ELISA plates prepared as described above. Aliquots (100 μl) of two-fold serially-diluted vaginal samples were used. HRP-conjugated anti-mouse IgA (Bethyl Laboratories; 1:10,000) or HRP-conjugated anti-mouse IgG (Promega, Sunnyvale, US) was used as the secondary antibody at a dilution of 1:2500.

Statistical analysis

Data were expressed as arithmetic mean ± standard deviation (mean ± SD). Data analysis was conducted using Student's *t*-test (EXCEL version 11.5, Microsoft). A *P*-value of <0.05 was considered significant.

Results

In vitro Expression of Env and hCD40Lm

We first confirmed the expression of the Env and hCD40Lm genes by the vectors constructed in this study by immunoblot assay. As shown in Fig. 1B, 293T cells transfected or infected with the recombinant Env expression plasmid pCAGGS-JRCSFrev/Env, or the Sendai virus (SeV) vector expressing HIV-1_{JR-CSF} envelope gene, produced gp120 and gp160. Four recombinant m8Δs were examined: m8Δ-Env carrying only the HIV-1_{JRCSF} envelope protein; m8Δ-Env/hCD40Lm carrying the envelope protein and hCD40Lm; and m8Δ-pSFJ1-10-hCD40Lm and m8Δ-p7.5-hCD40Lm, carrying hCD40Lm under different promoters, pSFJ1-10 and p7.5, respectively (Fig. 1B). Cells infected with the Env gene harboring m8Δs expressed mainly gp160 and lesser amounts of gp120 (Fig. 1B). To determine whether functional envelope proteins were produced, the m8Δ recombinants were used to infect TZM-bl cells that express CCR5 and CD4 and examined whether cell fusion could be provoked. We observed large fused TZM-bl cells, but HeLa cells infected with these viruses did not fuse (data not shown). These data indicate that functional gp120 and gp41 were produced. In cells infected with hCD40Lm harboring m8Δs, authentic hCD40Lm protein was produced and cells infected with m8Δ-pSFJ1-10-hCD40Lm expressed more CD40Lm than those infected with m8Δ-p7.5-hCD40Lm or m8Δ-Env/hCD40Lm (Fig. 1B). These data are consistent with the relative strong potency of the pSFJ1-10 promoter vs. the p7.5 promoter [40].

Effect of hCD40Lm in the Env expressing DNA prime/LC16m8Δ boost regime

The effect of CD40Lm in improving the immunogenicity of the vaccination regime was first examined using the most frequently tested regime of priming with an Env-expressing plasmid followed by boosting with Env-expressing vaccinia m8Δ (Table 1 group A–F). Female C57BL/6J mice were primed with 50 μg of pCAGGS-JRCSFrev/env followed by boosting with 1×10^7 PFU of various m8Δ recombinants: m8Δ (group A), m8Δ-Env (group B), m8Δ-Env/hCD40Lm (group C), m8Δ-Env and m8Δ (group D), m8Δ-Env and m8Δ-p7.5hCD40Lm (group E), and m8Δ-Env and m8Δ-pSFJ1-10hCD40Lm (group F) (Fig. 2A). Mice were sacrificed two weeks after the final immunization, and splenocytes and sera were collected for immunological assays. An ICS FACS analysis was conducted to identify HIV-1 Env-specific IFN-γ secreting $CD8^+$ T cells after stimulation with a consensus subtype B Env (15-mer) peptide pool. Initially, all overlapping Env peptides representing entire JR-CSF Env were tested for their ability to induce most effective response (frequency of IFN-γ secreting $CD8^+$ T cells), then the two most immunogenic peptides, 805–819 aa and 809–823 aa, were selected and the mixture of these two peptides was used thereafter. Co-immunization of m8Δ-Env with the lower expression hCD40Lm vector, m8Δ-p7.5hCD40Lm (group E), significantly enhanced the number of IFN-γ secreting $CD8^+$ T cells by approximately 2–4 fold compared to the groups boosted with m8Δ-JRCSFenv alone (group B) or m8Δ-JRCSFenv together with empty m8Δ (group D). In contrast, the higher expression hCD40Lm vector, m8Δ-pFSJ1-10hCD40Lm (group F), decreased the number of IFN-γ expressing $CD8^+$ T cells. There was no significant difference in the number of IFN-γ secreting $CD8^+$ T cells between mice boosted with the coexpression vector, m8Δ-Env/hCD40Lm (group C), and mice boosted with m8Δ-Env (group B) (Fig. 2B). IFN-γ and CD107a double positive $CD8^+$ T cell fractions were also quantified to determine the number of functional $CD8^+$ T cells. As in the case of IFN-γ secreting $CD8^+$ T cells, co-immunization with m8Δ-p7.5hCD40Lm enhanced the immunogenicity of m8Δ-env (data not shown). Interestingly, the expression of an optimal amount of hCD40Lm was important for the enhancement since high levels of expression were not effective (Compare group E and F in Fig. 2B). The m8Δ-pFSJ1-10hCD40Lm vector (group F) was therefore omitted from subsequent experiments. The data above suggest that uncleaved CD40L delivered by m8Δ vector help the Env expression pox vector to generate more effective T cell responses, but only when CD40Lm are expressed by the separate virus.

Although we tried to detect HIV-1_{JR-CSF} Env-specific antibodies by ELISA and TZM-bl cell-based assays, the DNA prime/m8Δ

boost immunization regimens did not elicit any anti-Env antibodies (see Fig. 4, group B as representative data).

Cellular immunity elicited in the Env expressing LC16m8Δ prime/SeV boost regimen

To identify vaccination methods that may elicit both cellular and humoral immunity, a novel immunization regimen using SeV and m8Δ expressing HIV-$1_{JR\text{-}CSF}$ Env was tested. At first we performed a preliminary experiment to optimize the order of prime-boost regime, in which, an m8Δ-Env prime/SeV-env boost regime produced better responses than a SeV-env prime/m8Δ-Env boost regime (data not shown). The priming effects of various combinations of m8Δ recombinants such as m8Δ-Env plus m8Δ-p7.5hCD40Lm (group G), m8Δ-Env plus m8Δ (group H), m8Δ-Env/hCD40Lm (group I), m8Δ-Env (group J), and m8Δ (group K) were examined; untreated mice were the control (group L) (Fig. 3A). A higher frequency of IFN-γ^+ $CD8^+$ T cells was detected in the m8Δ-Env prime/SeV-env boost regime than in the DNA prime/m8Δ-Env boost regimen (compare Fig. 2B and Fig. 3B). Immunization with the coexpression vector m8Δ-Env/hCD40Lm (group I) did not enhance the number of IFN-γ^+ $CD8^+$ T cells compared to m8Δ-Env alone (group J), similar to the DNA prime/m8Δ boost (see Fig. 2). In contrast, the group G, in which mice were primed with a combination of m8Δ-Env and m8Δ-p7.5hCD40Lm, significantly increased the frequency of Env-specific IFN-γ secreting $CD8^+$ T cells compared to the group that was primed with m8Δ-Env and empty m8Δ (group H, $p<0.005$) (Fig. 3B). A more detailed analysis of IFN-γ^+ $CD107a^+$ CD8+ T cells by comparison of groups G, H, I and K showed similar results in that m8Δ-p7.5hCD40Lm co-immunization with m8Δ-Env increased the number of functional $CD8^+$ T cells (data not shown). These results indicate that the m8Δ prime and SeV boost regime efficiently elicits Env-specific cellular immunity, and that the inclusion of hCD40Lm delivered by a separate vector enhances cellular immunity elicited by m8Δ-Env. We also compared the frequency of IFN-γ secreting cells in $CD4^+$ T cell populations (Fig. 3C). The $CD4^+$ T cells responses to Env peptides 805–819 aa and 809–823 aa, stimulation were not enhanced by hCD40Lm expressed by either the same or separate vector (compare group G, H and I). Although the frequencies of IFN-γ^+ $CD4^+$ T cells were much lower than those of $CD8^+$ T cells, all were significantly higher than empty m8Δ (group K). The relatively lower CD4+ T cell response might be due to the poor match of the peptides to the MHC-II molecules. Therefore, we performed epitope mapping using overlapping Env peptides that cover the entire Env protein, but no peptide induced more efficient $CD4^+$ T cell response than the two peptides used above (data not shown), ruling out the possibility that stimulation with Env peptides 805–819 aa and 809–823 aa did not faithfully reflect authentic $CD4^+$ T cell response.

Antibodies elicited in the Env expressing LC16m8Δ prime/SeV boost regimen

To determine whether anti-Env antibodies were elicited, ELISAs were conducted using gp160-coated plates. All regimens involving vaccinia virus prime/SeV boost efficiently elicited anti-Env antibodies (Fig. 4, group G–J), indicating that our novel immunization regimen of m8Δ prime/SeV boost is able to elicit both humoral and cellular immune responses. In contrast, the conventional immunization regimen of DNA prime/vaccinia virus boost elicited only cellular immunities, not antibody responses. Although priming with the coexpression vector m8Δ-Env/hCD40Lm (group I) did not enhance Env-specific cellular immunity, it did elicit significantly more anti-Env antibodies than m8Δ-Env alone. Co-immunization with m8Δ-Env and m8Δ-p7.5hCD40Lm (group G) did not significantly augment the anti-Env antibody production compared to priming with m8Δ-Env alone or m8Δ-Env plus m8Δ (group J and H). Comparison of the endpoint dilution titer of the antibodies, suggests that m8Δ-Env/hCD40Lm priming (group I) elicited approximately 5–6 times more anti-Env antibody than the other immunization groups (Fig. 4B).

Next, the avidity of the anti-Env antibodies was assessed based on the relative amount of antibody that remained on the ELISA plate after a urea wash (Fig. 5). The ED_{50} of the urea wash for the anti-Env antibodies prepared from mice primed with m8ΔEnv/hCD40Lm, m8ΔEnv + m8ΔhCD40Lm, m8ΔEnv and m8ΔEnv+m8Δ (group I, G, J and H) is 5.16 M, 4.22 M, 3.84 M and 2.57 M, respectively (Fig. 5A). The relative amount of residual anti-Env antibody from group I was significantly higher than any other group after washing with 7 M urea (Fig. 5B). These results indicate that priming with the coexpression vector m8Δ-Env/hCD40Lm markedly enhanced the avidity of the Env antibodies produced, compared to the Env expression vector alone; co-immunization with m8Δ-Env and m8Δ-p7.5hCD40Lm (group G) did not enhance avidity.

Finally, the titer of neutralizing antibodies was determined using pseudotyped viruses coated with gp160 of JR-CSF, tier 1 SF162, and several tier 2 viruses belonging to clade B and C using TZM-bl reporter cells [35,43]. All m8Δ prime/SeV boost regimens except the control groups K and L elicited neutralizing antibodies against SF162: the mean titers (ID_{50}) of the sera prepared from mice primed with m8Δ-Env/hCD40Lm, m8Δ-Env+m8Δ-hCD40Lm, and m8Δ-Env (group I, G and J) were 954, 287, and 205, respectively. The m8Δ-Env/hCD40Lm-primed group I exhibited a significant enhancement of neutralizing antibody titer against SF162 (Fig. 6). Co-immunization with m8Δ-Env and m8Δ-p7.5hCD40Lm (group G) did not show significantly enhanced nAbs to SF162. Sera from untreated mice contained low levels of a non-specific inhibitor to SF162 (titer approximately 100), but the level is insignificant. However, these regimens, irrespective of the inclusion of hCD40Lm, were not effective in eliciting nAbs against tier 2 viruses, including JR-CSF. Immunization twice with the plasmid expressing gp160 followed by boosting with SeV-env did not elicit detectable anti-Env antibody (see Fig. 4), indicating the importance of priming with m8Δ-Env. Priming and boosting with the same m8Δ-Env did not elicit significant titers of nAb (data not shown). To further confirm that the antibodies but not other factors were responsible for the neutralizing activity of the sera, we purified antibodies using protein A Sepharose. All the purified antibodies showed IgG isotypes. We performed neutralizing antibody measurement using TZM-bl reporter cells as described above. The neutralization capacities of purified immunoglobilins showed high identicalness with the neutralizing titer of original sera ($R^2 = 0.777$) (Fig. 6B) confirming that neutralizing antibodies were elicited using our m8Δ prime/SeV boost regimens.

An immunological correlate analysis was conducted using Spearman rank correlates to evaluate the relationship between the SF162 neutralization capacity and the avidity of JR-CSF Env binding antibodies; a significant positive correlation was present ($R = 0.686$, $P<0.0001$; Fig. 7A). A weaker, but still significant, positive correlation between the nAb titers and the amount of the Env-binding antibodies was identified ($R = 0.501$, $P = 0.0013$; Fig. 7B). Moreover, the amounts of Env-binding antibodies showed a positive correlation with the antibody avidity ($R = 0.555$, $P = 0.000377$; Fig. 7C). These results verify that better immunized mice produced not only greater amounts of antibody,

but also higher affinity antibodies with enhanced neutralizing capacity.

Secretion of anti-Env IgG and IgA into vaginal fluid was examined. Because of the limited amounts of mouse vaginal fluid, fluid from 5–6 mice was mixed for these assays. The amounts of total IgG in the fluid were relatively constant. Fluid prepared from the DNA prime/SeV-env boost group contained 277 ng/ml IgG, the m8Δ-Env prime/SeV-env boost group contained 270 ng/ml, the m8Δ-Env/hCD40Lm prime/SeV-env boost group contained 252 ng/ml, and the m8Δ-Env + m8Δ-hCD40Lm prime/SeV-env group contained 242 ng/ml. Similar levels of anti-gp160 IgG were detected in all the m8Δ primed groups, but no gp160-specific IgG was detected in the DNA primed group (Fig. 8). Anti-Env IgA was not detected in significant amounts in any immunization group (data not shown).

Discussion

Although researchers have proposed either antibodies or T cells as the most effective means to elicit protective immunity, a central theme of HIV-1 vaccine design now is to elicit coordinated antiviral $CD8^+$ cytotoxic T lymphocytes (CTL) to control HIV-1 infection and $CD4^+$ T cells that help induce and maintain $CD8^+$ and B cell responses [44–46]. In this study, we found that a novel immunization schedule including a HIV-1 Env expressing m8Δ prime/SeV boost regimen is able to elicit both Env-specific $CD8^+$ T cells and antibodies. These results sharply contrast the results of conventional DNA prime/virus vector boost regimens that have elicited $CD8^+$ T cell responses with poor antibody response, which is consistent with the previous report [47]. Moreover, this regime elicited anti-Env IgG secreted into the vaginal fluid. Thus, the m8Δ prime/SeV boost regimen should provide a basis for an improved immunization protocol. Although nAbs against tier 2 viruses were absent, recent reports have suggested that non-neutralizing Env binding antibodies play a role in preventing infection by HIV-1/SIV [9,48]. Thus, the humoral immunity elicited by the m8Δ prime/SeV boost regimen may also have an improved efficacy against HIV-1 infection. It is an important future theme to elicit broad nAbs against tier 2 viruses on the basis of this regimen.

In m8Δ prime/SeV boost regimen only priming with m8Δ-Env did not elicit any anti-Env antibody before rSeV boost (data not shown), indicating the necessity of rSeV booster. Intranasal Sendai virus was well tolerated and had good immunogenicity [19], making it a good candidate for HIV vaccine. Indeed, involvement of rSeV in prime-boost-boost HIV vaccine strategies had been reported to elicit persistent humoral response in BALB/c mice and rhesus macaques [49] as well as in pre-clinical trials [50]. However, no elicitation of neutralizing antibody has been reported. Our results that m8Δ prime/SeV boost in combination with hCD40Lm adjuvant regimen efficiently elicited neutralizing antibody suggests that this regimen may be a better vaccine strategy.

Recent advances in immunology have shown that several types of molecules may be used as novel adjuvants to enhance the immunogenicity of vaccines. In addition to alum and MF59, other adjuvants including cytokines, chemokines, toll-like receptor ligands and some co-stimulatory molecules may have potential for clinical use [51–54]. Among these molecules, CD40L is one of the most potent stimuli for DCs, which activate CTL and B cells [15,25–28]. We showed that hCD40Lm expressing m8Δ in conjunction with Env expressing vaccinia virus enhanced production of HIV-1 specific CTL, but not antibodies. In contrast, the coexpression m8Δ-Env/hCD40Lm vector did not increase the induction of Env-specific $CD8^+$ T cells compared to m8Δ-Env alone, but did elicit more anti-Env antibodies with higher avidity and neutralizing capacity against tier 1 SF162. The high avidity antibodies elicited by m8Δ-Env/hCD40Lm may constitute nAb. Indeed, we found a positive correlation between the avidity of Env-binding antibodies and neutralizing activity against HIV-1 SF162 (Fig. 7). The enhancement of antiviral immunity by hCD40Lm in this mouse model also suggests that human CD40Lm is functional in mouse, encouraging us to use it as an AIDS vaccine adjuvant. We have to admit that our novel vaccine regimen still has room to be improved. The species variation between human and mouse CD40Lms should be considered when noting that the enhancement of humoral immunity to be less impressive. Using homogenous CD40Lm or further inclusion of other adjuvant is encouraged to magnitude antibody response of our novel vaccine regimen. Although the reason for the divergent effects of hCD40Lm expressed from different constructs on the production of viral specific immunity is unclear, Given that co-immunization with the two vectors may result in gp160 and CD40Lm being expressed on the same cell or on different cells, whereas immunization with the coexpression vector m8Δ-RCSFenv/hCD40Lm results in gp160 and CD40Lm being expressed on the same cells. Simultaneous expression of gp160 and CD40Lm may preferentially stimulate B cells through a direct interaction with the vector-infected cells because only B cells express both CD40 and a B cell receptor to gp160. The expression of CD40Lm alone may promote the maturation of DCs that were sensitized by gp160 and lead to the activation of cellular immunity. Alternatively, the expression levels of Env and hCD40Lm in cells that have been infected with both m8Δ-Env and m8Δ-hCD40Lm may be different from levels in cells infected with m8Δ-Env/hCD40Lm, leading to different immune responses. Other hypothesis to explain these phenomena cannot be excluded at this point.

The detection of anti-Env IgG in vaginal fluid indicates that our vaccine regimen can also elicit mucosal immunity. However, in contrast to the enhancing effect of hCD40Lm on production of anti Env antibodies in sera, we didn't find that inclusion of hCD40Lm promote levels of anti-gp160 IgG in vaginal fluid as seen in Fig. 8. The reason for this difference is currently under elucidation. Further investigation of mechanism responsible for this divergent effect may be profitable to improve our vaccine regimen to achieve more potent mucosal immunity against HIV.

Recently, monoclonal antibodies (mAbs) that broadly neutralize most HIV-1 strains have been isolated from chronically infected subjects, and analyses of the epitopes recognized by these antibodies may direct the way to devise antigens that elicit broad nAbs [55–57]. These studies suggest the possibility of a vaccine that elicits the production of broadly neutralizing antibodies that could prevent HIV-1 infection. However, detailed analyses of broadly neutralizing mAbs have indicated a requirement for extensive affinity maturation of the cognate immunoglobulin genes [57]. The observation that broad neutralizing Abs are generated after a long incubation period in HIV-1-infected individuals also supports this idea and, furthermore, suggests the importance of repetitive antigenic stimulation [58,59]. Therefore, in addition to devising specific antigens, the development of methods to promote the affinity maturation of antibodies and maturation of B cells should be equally important. This study has demonstrated that CD40Lm, which can activate class switching of immunoglobulin genes and maturation of dendritic cells [22,23], is suitable for eliciting more potent nAbs. In addition, replication-competent m8Δ that repeatedly presents native antigens in vivo may be important for effective immunization.

In conclusion, this study showed that a novel vaccine regimen that includes the expression of hCD40Lm in the context of LC16m8Δ priming and Sendai virus vector boosting was able to elicit both HIV-1 Env-specific cellular and humoral immunities. Thus, such a regimen may provide a platform for HIV-1 vaccine development, as well as other infectious pathogens.

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Author Contributions

Conceived and designed the experiments: XZ H. Shida. Performed the experiments: XZ T. Sobue M. Isshiki SM H. Sato. Analyzed the data: XZ T. Sobue M. Isshiki SM H. Shida. Contributed reagents/materials/analysis tools: M. Inoue KK T. Shioda TO JK HH. Wrote the paper: XZ H. Shida.

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The Carboxyl-Terminus of Human Immunodeficiency Virus Type 2 Circulating Recombinant form 01_AB Capsid Protein Affects Sensitivity to Human TRIM5α

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Abstract

Human immunodeficiency virus (HIV) type 2 shows limited geographical distribution compared with HIV type 1. Although 8 genetic groups of HIV type 2 (HIV-2) have been described, recombinant viruses between these groups are rarely observed. Recently, three HIV-2 patients in Japan were described with rapidly progressive, acquired immunodeficiency. These patients were infected with an A/B inter-group recombinant designated CRF01_AB. Here, we characterize the capsid protein (CA) encoded by the viruses from these patients. HIV-2 CRF01_AB CA showed unique amino acid sequence almost equally distinct from group A and group B viruses. Notably, HIV-2 CRF01_AB CA showed potent resistance to human TRIM5α. In addition to the previously identified amino acid position 119 in the N-terminal domain of CA, we found that HIV-2 CRF01_AB-specific amino acid substitutions in the C-terminal domain also were necessary for resistance to human TRIM5α. These results indicate that retroviruses can evade TRIM5α by substitution at residues within the C-terminal domain of CA.

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Introduction

Human immunodeficiency virus type 2 (HIV-2) has been detected primarily in West Africa, in contrast to the global distribution of the type 1 epidemic virus (HIV-1). Based on molecular evidence, HIV-2 and HIV-1 are presumed to derive from simian immunodeficiency viruses that originated in sooty mangabey (SIVsm) and chimpanzee (SIVcpz), respectively, as a result of zoonotic transfer between non-human primates and human. The HIV-1 and HIV-2 bear a considerable degree of homology in both gene organization and RNA sequence (30%–60%) [1–4]. It is generally believed that HIV-2 is less pathogenic than HIV-1. However, certain HIV-2 patients with high plasma HIV-2 loads develop acquired immune deficiency syndrome (AIDS) as rapidly as HIV-1 patients do [4]. To date, eight HIV-2 groups have been distinguished on the basis of phylogenetic (sequence) analysis; each group is presumed to have originated from an independent zoonotic event [5].

TRIM5α was identified as a factor that restricts HIV-1 infection in rhesus monkey (Rh) cells [6]. TRIM5α is thought to degrade the core of the incoming virus [7,8]. TRIM5 proteins are members of the tripartite motif family containing RING, B-box, and coiled-coil domains. The alpha isoform of TRIM5 has an additional C-terminal PRYSPRY (B30.2) domain [9]. In cynomolgus monkey (CM), TRIM5α also has been demonstrated to restrict HIV-1 infection [6,10]. In contrast, the human TRIM5α exhibits minimal restriction of HIV-1 infection [11–14], but shows moderate levels of restriction for HIV-2 [15].

Capsid (CA) proteins are components of the viral core; the CAs of HIV-1 and HIV-2 have similar primary and three dimensional structures [16]. CA is composed of a surface-exposed N-terminal domain (NTD) and a C-terminal domain (CTD) that is required for oligomerization [17]. We previously identified a single amino acid of the HIV-2 capsid that determines the susceptibility of HIV-2 to CM TRIM5α. Viruses that encoded CAs with either alanine or glutamine at amino acid residue 119 (which corresponded to the 120th amino acid of the CA of the GH123 viral strain) could grow in cells harboring the CM TRIM5α. In contrast, HIV-2 encoding CA with proline at the same position showed restricted growth in cells harboring the CM TRIM5α. Similar results, although to a lesser extent, were observed when the human TRIM5α was used [15]. Furthermore, an analysis of HIV-2 CA variation in a West African Caio cohort demonstrated that the presence of proline at CA positions 119, 159, and 178 was more frequent in individuals with lower viral loads (VLs); the presence of non-proline residues at all 3 residues was more frequent in