JOURNAL OF VIROLOGY, Oct. 2008, p. 10199–10206 Vol. 82, No. 20
0022-538X/08/$08.00+0 doi:10.1128/JVI.01103-08

Gag-Specific Cytotoxic T-Lymphocyte-Based Control of Primary Simian Immunodeficiency Virus Replication in a Vaccine Trial[▽]

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Received 26 May 2008/Accepted 21 July 2008

Gag-specific cytotoxic T lymphocytes (CTLs) exert strong suppressive pressure on human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) replication. However, it has remained unclear whether they can actually contain primary viral replication. Recent trials of prophylactic vaccines inducing virus-specific T-cell responses have indicated their potential to confer resistance against primary SIV replication in rhesus macaques, while the immunological determinant for this vaccine-based viral control has not been elucidated thus far. Here we present evidence implicating Gag-specific CTLs as responsible for the vaccine-based primary SIV control. Prophylactic vaccination using a Gag-expressing Sendai virus vector resulted in containment of SIVmac239 challenge in all rhesus macaques possessing the major histocompatibility complex (MHC) haplotype *90-120-Ia*. In contrast, *90-120-Ia*-positive vaccinees failed to contain SIVs carrying multiple *gag* CTL escape mutations that had been selected, at the cost of viral fitness, in SIVmac239-infected *90-120-Ia*-positive macaques. These results show that Gag-specific CTL responses do play a crucial role in the control of wild-type SIVmac239 replication in vaccinees. This study implies the possibility of Gag-specific CTL-based primary HIV containment by prophylactic vaccination, although it also suggests that CTL-based AIDS vaccine efficacy may be abrogated in viral transmission between MHC-matched individuals.

Despite tremendous efforts to develop AIDS vaccines eliciting virus-specific T-cell responses, whether this approach actually does result in controlling human immunodeficiency virus (HIV) replication remains unknown. Recent trials have shown reductions in postchallenge viral loads by prophylactic vaccination eliciting virus-specific T-cell responses in macaque AIDS models (19, 22, 34), but the first advanced human trial of a T-cell-based vaccine was halted because of a lack of efficacy (5). Hence, it is quite important to determine which T-cell responses are responsible for primary HIV control.

Cytotoxic T-lymphocyte (CTL) responses have been indicated to play an important role in the control of HIV and simian immunodeficiency virus (SIV) infections (2, 9, 10, 17, 23, 29). Above all, the potential of Gag-specific CTL responses to contribute to viral control has been suggested by a cohort study indicating an association of HIV control with the breadth of Gag-specific CTL responses (15). In support of this, a recent in vitro study revealed their ability to rapidly respond to SIV infection (28). However, it has remained unclear whether Gag-specific CTL-based viral containment can be achieved by prophylactic vaccination.

We previously developed a prophylactic AIDS vaccine regimen consisting of a DNA prime followed by a boost with a Sendai virus (SeV) vector expressing SIVmac239 Gag (SeV-Gag) (22, 32). Our trial showed potential for efficiently inducing Gag-specific T-cell responses and containment of SIVmac239 challenge in a group of Burmese rhesus macaques sharing the major histocompatibility complex class I (MHC-I) haplotype *90-120-Ia* (22). A follow-up study revealed the reappearance of plasma viremia at >1 year postchallenge in some of these *90-120-Ia*-positive SIV controllers. In these transient controllers, multiple CTL escape mutations were accumulated in the viral *gag* gene, resulting in viremia reappearance and thus suggesting the involvement of $Gag_{206-216}$ (IINEEAADWDL) epitope-specific, $Gag_{241-249}$ (SSVDEQIQW) epitope-specific, and $Gag_{373-380}$ (APVPIPFA) epitope-specific CTLs in sustained viral control (12). Nonetheless, it has remained undetermined whether such Gag-specific CTL responses were responsible for the vaccine-based primary SIV control in *90-120-Ia*-positive vaccinees. In the present study, we challenged the *90-120-Ia*-positive vaccinees with SIVs carrying the *gag* CTL escape mutations to determine the role of Gag-specific CTLs in primary SIVmac239 control.

MATERIALS AND METHODS

Viral competition assay. SIV molecular clone DNAs with *gag* mutations were constructed by site-directed mutagenesis from the wild-type SIVmac239 (14) molecular clone DNA. Virus stocks were obtained by transfection of COS-1 cells with wild-type or mutant SIV molecular clone DNAs, and their titers were

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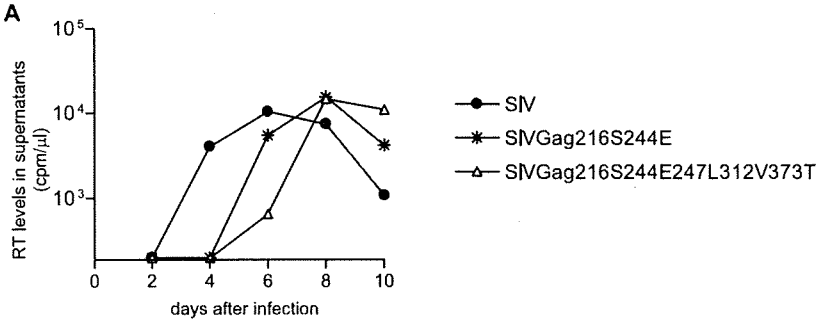
▽ Published ahead of print on 30 July 2008.



B

Competition between SIVmac239 vs SIVmac239Gag216S244E

inoculated dose ratio 1/1

	day 6	day 18
Exp 1	Wt	Wt
Exp 2	Wt	Wt
Exp 3	Wt (mt)	Wt

Competition between SIVmac239Gag216S244E vs SIVmac239Gag216S244E247L312V373T

	day 6	day 18
Exp 1	Wt	Wt
Exp 2	Wt (mt)	Wt
Exp 3	Wt (mt)	Wt

C

inoculated dose ratio 1/4

	day 6	day 18
SIVmac239	6/13	15/16
SIVmac239Gag216S244E	7/13	1/16

	day 6	day 18
SIVmac239Gag216S244E	7/18	12/16
SIVmac239Gag216S244E247L312V373T	11/18	3/16
*SIVmac239Gag216S244E312V373T		1/16

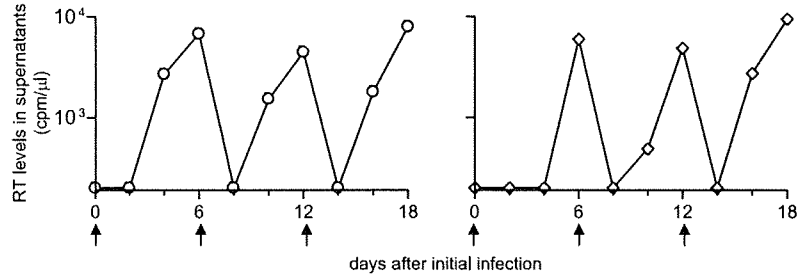


FIG. 1. Replication of mutant SIVs in vitro. (A) Wild-type and mutant SIV replication kinetics in HSC-F cells. HSC-F cells were infected with SIVmac239 (closed circles), SIVmac239Gag216S244E (asterisks), or SIVmac239Gag216S244E247L312V373T (open triangles). Virus production was monitored by measuring RT activity in the culture supernatants. Representative results from three sets of experiments are shown. (B) Viral competition assay. HSC-F cells were coinfected with SIVmac239 and SIVmac239Gag216S244E (left) or with SIVmac239Gag216S244E and SIV mac239Gag216S244E247L312V373T (right) at a ratio of 1:1. Viral *gag* fragments were amplified by RT-PCR from viral RNAs from the culture supernatants at days 6 and 18 postinfection and then sequenced. Dominant amino acid sequences at the 216th and 244th aa (left) or the 247th, 312th, and 373rd aa (right) in Gag in three sets of experiments are shown. Wt, only the wild-type sequence was detected; Wt (mt), the wild type was dominant, but the mutant was detectable (the mutant/wild-type ratio was <1/2). (C) Viral competition assay. HSC-F cells were coinfected with SIVmac239 and SIVmac239Gag216S244E (left) or with SIVmac239Gag216S244E and SIVmac239Gag216S244E247L312V373T (right) at a ratio of 1:4. The amplified *gag* fragments were subcloned into plasmids and sequenced. Frequencies of the indicated SIV clones (number of indicated clone per total number of clones) are shown. Changes in RT levels in the culture supernatants are shown in the bottom panels. The arrows indicate the time points of coinfection (at day 0) and viral passage for the second (at day 6) and the third (at day 12) cultures.

measured by reverse transcription (RT) assay as described previously (25, 33). For analysis of viral replication, HSC-F cells (herpesvirus saimiri-immortalized macaque T-cell line) (1) were infected with wild-type or mutant SIVs (normalized by RT activity), and virus production was monitored by measuring RT activity in the culture supernatants. For competition, HSC-F cells were coinfected with two SIVs at a ratio of 1:1 or 1:4, and the culture supernatants were harvested every other day and used for RT assays. On day 6, the supernatant was added to fresh HSC-F cells to start the second culture. Similarly, on day 12 after the initial coinfection, the second culture supernatant was added to fresh HSC-F cells to start the third culture. RNAs were extracted from the initial culture supernatant on day 6 and from the third culture supernatant on day 18 postcoinfection. The fragment (nucleotides 1231 to 2958 in SIVmac239 [GenBank accession number M33262]) containing the entire *gag* region was amplified from the RNA by RT-PCR and sequenced. Alternatively, it was subcloned into plasmids to determine dominant sequences.

Animal experiments. Burmese rhesus macaques (*Macaca mulatta*) were maintained in accordance with the guidelines for animal experiments performed at the National Institute of Infectious Diseases (26). Three animals, R01-007, R02-003, and R02-012, that received a prophylactic DNA prime/SeV-Gag boost vaccine and contained SIVmac239 challenge have been reported previously (22). In the present study, macaques R06-015, R06-035, R06-041, R05-004, R05-027, and R07-005 also received the DNA prime/SeV-Gag boost vaccine. The DNA used for the vaccination, CMV-SHIVdEN, was constructed from *env*- and *nef*-deleted simian-human immunodeficiency virus $SHIV_{MD14YE}$ molecular clone

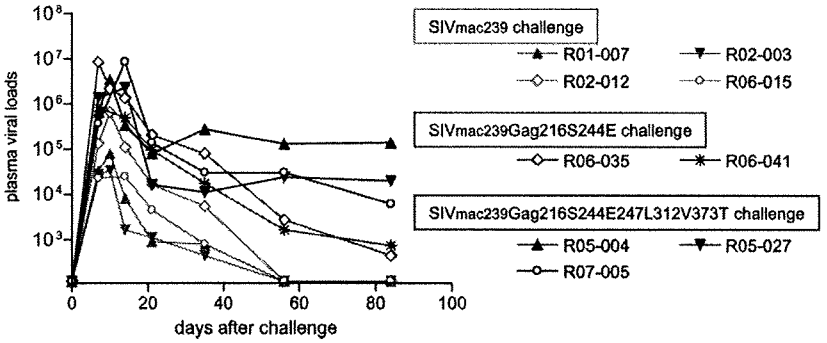


FIG. 2. Plasma viral loads after wild-type or mutant SIV challenge. The *90-120-Ia*-positive vaccinees were challenged with SIVmac239 (red lines), SIVmac239Gag216S244E (blue lines), or SIVmac239Gag216S244E247L312V373T (black lines). Plasma viral loads (SIV *gag* RNA copies/ml plasma) were determined as described before (22). The lower limit of detection is approximately 4×10^2 copies/ml.

DNA (SIVGP1) (31, 32) and has the genes encoding SIVmac239 Gag, Pol, Vif, and Vpx, SIVmac239-HIV chimeric Vpr, and HIV Tat and Rev. At the DNA vaccination, animals received 5 mg of CMV-SHIVdEN DNA intramuscularly. Six weeks after the DNA prime step, animals received a single boost intranasally with 6×10^9 cell infectious units of F-deleted replication-defective SeV-Gag (21, 32). Approximately 3 months after the boost, animals were challenged intravenously with 1,000 50% tissue culture infective doses of SIVmac239, SIVmac239 Gag216S244E, or SIVmac239Gag216S244E247L312V373T. The challenge virus stocks were prepared by virus propagation on rhesus macaque peripheral blood mononuclear cells (PBMCs). Sequence analysis confirmed the absence of *gag* mutations except for the two or five mutations in the challenge viruses.

Immunostaining of $CD4^+$ T-cell memory subsets. PBMCs were subjected to immunofluorescence staining by using fluorescein isothiocyanate-conjugated anti-human CD28, phycoerythrin-conjugated anti-human CD95, peridinin chlorophyll protein-conjugated anti-human CD4, and allophycocyanin-conjugated anti-human CD3 monoclonal antibodies (Becton Dickinson, Tokyo, Japan). The central memory subset of $CD4^+$ T cells was defined by possession of a $CD28^+$ $CD95^+$ phenotype, as described previously (13, 27).

Measurement of virus-specific $CD8^+$ T-cell responses. We measured virus-specific $CD8^+$ T-cell levels by flow cytometric analysis of gamma interferon (IFN-γ) induction after specific stimulation, as described previously (13, 22). In brief, PBMCs were cocultured with autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines infected with a vaccinia virus vector expressing SIVmac239 Gag for Gag-specific stimulation or a vesicular stomatitis virus G protein-pseudotyped SIVGP1 for SIV-specific stimulation. The pseudotyped virus was obtained by cotransfection of COS-1 cells with a vesicular stomatitis virus G protein expression plasmid and the SIVGP1 DNA. Alternatively, B-lymphoblastoid cell lines were pulsed with 1 to 10 μM peptides for peptide-specific stimulation (11, 12). The 15-mer $Gag_{367\text{-}381}$ peptide was used to detect $Gag_{367\text{-}381}$-specific CTLs, including $Gag_{373\text{-}380}$-specific CTLs. Intracellular IFN-γ staining was performed using a Cytofix Cytoperm kit (Becton Dickinson). Peridinin chlorophyll protein-conjugated anti-human CD8, allophycocyanin-conjugated anti-human CD3, and phycoerythrin-conjugated anti-human IFN-γ antibodies (Becton Dickinson) were used. Specific T-cell levels were calculated by subtracting nonspecific IFN-γ^+ T-cell frequencies from those after Gag-specific, SIV-specific, or peptide-specific stimulation. Specific T-cell levels of <100 cells per million PBMCs were considered negative.

Statistical analysis. Statistical analysis was performed with Prism software, version 4.03, with significance set at P values of <0.05 (GraphPad Software, Inc., San Diego, CA). Central memory $CD4^+$ T-cell counts before challenge were not significantly different between the wild-type SIV-challenged ($n = 4$) and the mutant SIV-challenged ($n = 5$) macaques ($P = 0.70$ by unpaired two-tailed t test with Welch's correction and $P = 0.73$ by nonparametric Mann-Whitney U test). Ratios of the central memory $CD4^+$ T-cell counts from a few months postchallenge to those prechallenge were log transformed and compared between the two groups by an unpaired two-tailed t test and the Mann-Whitney U test. Gag-specific $CD8^+$ T-cell frequencies postvaccination (prechallenge) or postchallenge were also log transformed and compared between the two groups in the same statistical manner.

RESULTS

Comparison of viral fitness in wild-type and mutant SIVs. We used two mutant SIVs for challenge of the *90-120-Ia*-positive vaccinees. The first, designated SIVmac239Gag216S244E, carries two *gag* mutations, GagL216S and GagD244E, leading to a leucine (L)-to-serine (S) substitution at the 216th amino acid (aa) and an aspartic acid (D)-to-glutamic acid (E) substitution at the 244th aa in Gag. The second, designated SIVmac239Gag216S244E247L312V373T, carries five *gag* mutations, GagL216S, GagD244E, GagI247L (isoleucine [I] to L at the 247th aa), GagA312V (alanine [A] to valine [V] at the 312th aa), and GagA373T (A to threonine [T] at the 373rd aa). In our previous study (12), the former became dominant in the early phase (at approximately 4 months postchallenge) during the period of viral control, and the latter was dominant at viremia reappearance in a transient controller. GagL216S, GagD244E and GagI247L, and GagA373T mutations result in viral escape from recognition by $Gag_{206\text{-}216}$-specific, $Gag_{241\text{-}249}$-specific, and $Gag_{373\text{-}380}$-specific CTLs, respectively, while it remains unclear whether GagA312V was selected for by CTLs.

We first compared viral fitness in wild-type and mutant SIVs. In HSC-F cells (a macaque T-cell line), not only the wild type but also the mutant SIVs were able to replicate, but SIVmac 239Gag216S244E replication was less efficient than that of wild-type SIVmac239, and SIVmac239Gag216S244E247L312 V373T replication was even less efficient (Fig. 1A). In competitions between two SIVs, HSC-F cells were coinfected with both viruses, and viral genome sequences in the culture supernatants were assessed to establish which SIV became predominant. In culture supernatants of HSC-F cells after coinfection with SIVmac239 and SIVmac239Gag216S244E inoculated at a ratio of 1:1, the wild type rapidly became dominant (at day 6) (Fig. 1B). Coinfection at a ratio of 1:4 resulted in equivalence at day 6, but the wild type again dominated by day 18 (Fig. 1C). These results indicate a lower replicative ability of SIVmac239 Gag216S244E than of wild-type SIVmac239. In addition, competition between SIVmac239Gag216S244E and SIVmac239 Gag216S244E247L312V373T showed the lower replicative ability of the latter (Fig. 1B and C).

Challenge of *90-120-Ia*-positive vaccinees with wild-type or mutant SIVs. Next, we challenged *90-120-Ia*-positive macaques

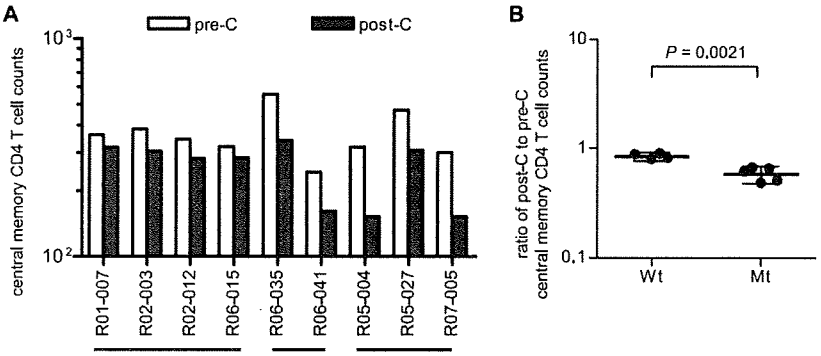


FIG. 3. Changes in central memory $CD4^+$ T-cell counts after wild-type or mutant SIV challenge. (A) Peripheral central memory $CD4^+$ ($CD4^+$ $CD95^+$ $CD28^+$) T-cell counts (/μl) prechallenge (pre-C) and a few months postchallenge (post-C). (B) Statistical comparison of central memory $CD4^+$ T-cell loss between the wild-type SIV-challenged (Wt) and the mutant SIV-challenged (Mt) macaques. The ratios of central memory $CD4^+$ T-cell counts postchallenge to those prechallenge are plotted. The longer bars indicate geometric mean values, and the regions between the shorter bars indicate the 95% confidence intervals. The ratios in the mutant group (n = 5) were significantly lower than those in the wild-type group (n = 4) (P = 0.0021 by unpaired t test and P = 0.0159 by Mann-Whitney U test).

with the mutant SIVs after DNA prime/SeV-Gag vaccination. Remarkably, all three vaccinees (R05-004, R05-027, and R07-005) challenged with SIVmac239Gag216S244E247L312V373T failed to control viral replication and showed high set point plasma viral loads, while all four vaccinees (R01-007, R02-003, R02-012, and R06-015) challenged with wild-type SIVmac239 contained viral replication, with undetectable set point plasma viral loads (Fig. 2). Even the two vaccinees (R06-035 and R06-041) challenged with SIVmac239Gag216S244E failed to contain viral replication, although with lower plasma viral loads, at approximately 10^3 RNA copies/ml at 3 months postchallenge. Central memory $CD4^+$ T-cell counts before challenge were not significantly different between the wild-type SIV-challenged (n = 4) and mutant SIV-challenged (n = 5) macaques, but ratios of the counts at a few months postchallenge to prechallenge for the latter group were significantly lower than those for the former (P = 0.0021 by unpaired t test and P = 0.0159 by Mann-Whitney U test) (Fig. 3). Thus, *90-120-Ia*-positive vaccinees can contain wild-type SIVmac239 but not SIVmac239Gag216S244E or SIVmac239Gag216S244 E247L312V373T challenge.

Viral *gag* sequence analysis confirmed the rapid selection for the GagL216S mutation in all wild-type SIVmac239-challenged macaques, as described previously (22). All of the *gag* mutations in the challenge mutant viruses were maintained during the observation period (Table 1). SIVmac239Gag216S244E247L312V373T-challenged macaques showed no additional dominant *gag* mutations, whereas animals challenged with SIVmac239Gag216S244E rapidly selected viruses with a GagV145A (V to A at the 145th aa) mutation. Recovery of viral fitness by this mutation was not observed, and whether it was selected for by CTLs was unclear in our previous study (12).

Gag-specific CTL responses were induced after SeV-Gag boost in all vaccinees, and there was no significant difference in the levels between the wild-type and mutant challenges (P = 0.1198 by unpaired t test and P = 0.1111 by Mann-Whitney U test). However, secondary Gag-specific CTL responses were

TABLE 1. Dominant sequences in SIV Gag in macaques after challenge

Macaque	Time (wk) of plasma sample	Amino acid change in Gag at position[a]:								
		140	145	206	216	244	247	312	341	373
R01-007	5				L216S					
R02-003	5				L216S					
R02-012	5				L216S					
R06-015	5			(I206M)	L216S					
R06-035	5				L216S*	D244E*				
	12		V145A		L216S*	D244E*			(N341Y)	
R06-041	5		(V145A)		L216S*	D244E*				
	12		V145A		L216S*	D244E*				
R05-004	5				L216S*	D244E*	I247L*	A312V*		A373T*
	12	(I140V)			L216S*	D244E*	I247L*	A312V*		A373T*
R05-027	5				L216S*	D244E*	I247L*	A312V*		A373T*
	12				L216S*	D244E*	I247L*	A312V*		A373T*
R07-005	5				L216S*	D244E*	I247L*	A312V*		A373T*
	12				L216S*	D244E*	I247L*	A312V*		A373T*

[a] A fragment containing the entire *gag* region was amplified from plasma RNA by nested RT-PCR and then sequenced. We were unable to amplify the fragment from plasmas obtained at week 12 from the wild-type SIVmac239-challenged macaques with undetectable viremia. Dominant *gag* mutations resulting in amino acid changes are shown. Asterisks indicate the mutations included in the challenge inoculums. Parentheses indicate that both the wild-type and mutant sequences were detected equivalently at that position.

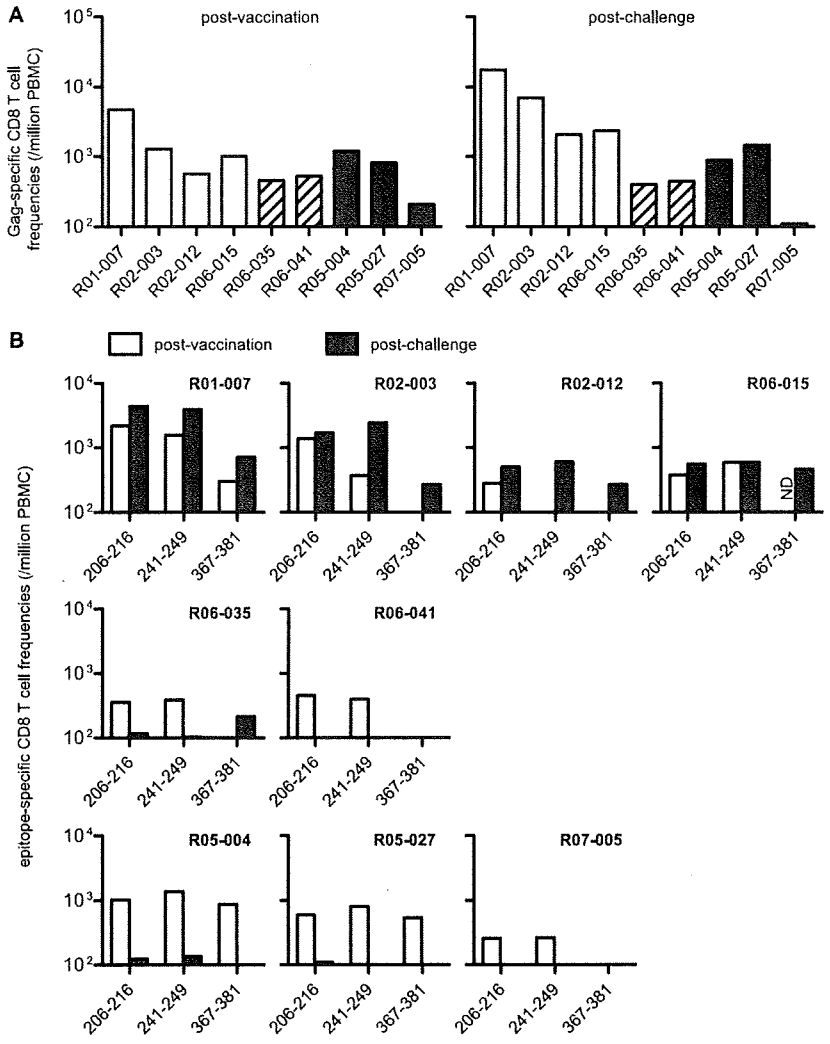


FIG. 4. Gag-specific $CD8^+$ T-cell responses before and after wild-type or mutant SIV challenge. Macaques R01-007, R02-003, R02-012, and R06-015 were challenged with SIVmac239; macaques R06-035 and R06-041 were challenged with SIVmac239Gag216S244E; and macaques R05-004, R05-027, and R07-005 were challenged with SIVmac239Gag216S244E247L312V373T. (A) Gag-specific $CD8^+$ T-cell frequencies at 2 weeks postboost (postvaccination) (left) and 2 weeks postchallenge (right). (B) $Gag_{206-216}$-specific, $Gag_{241-249}$-specific, and $Gag_{367-381}$-specific $CD8^+$ T-cell frequencies at 2 weeks (all except for R02-012) or 4 weeks (in R02-012) postboost (postvaccination) and 5 weeks (in R01-007, R02-003, R02-012, R06-035, R06-041, and R05-004) or 6 weeks (in R06-015, R05-027, and R07-005) postchallenge. ND, not determined.

less efficient after challenge with mutant SIV than after challenge with wild-type SIV ($P = 0.0095$ by unpaired t test and $P = 0.0159$ by Mann-Whitney U test) (Fig. 4A).

SeV-Gag. boost induced efficient $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses in all vaccinees and $Gag_{367-381}$-specific CTL responses in some of them (Fig. 4B). Challenge with wild-type SIVmac239 resulted in efficient secondary responses of these three epitope-specific CTLs, whereas SIVmac 239Gag216S244E247L312V373T challenge evoked none of them (Fig. 4B). SIVmac239Gag216S244E challenge did not result in secondary responses of $Gag_{206-216}$-specific or $Gag_{241-249}$-specific CTLs but did induce $Gag_{367-381}$-specific CTL responses in one case (Fig. 4B). These results indicate that SIVmac239 Gag216S244E evades recognition by $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTLs and that SIVmac239Gag216S244E2 47L312V373T evades recognition by $Gag_{206-216}$-specific, $Gag_{241-249}$-specific, and $Gag_{367-381}$-specific CTLs.

We next examined Gag-specific and SIV-specific CTL responses after mutant SIV challenge (Fig. 5A). We used an *env*- and *nef*-deleted SHIV molecular clone DNA, SIVGP1, that has the genes encoding SIVmac239 Gag, Pol, Vif, Vpx, and a part of Vpr and measured the frequencies of CTLs responding to SIVGP1-transduced cells (referred to as SIV-specific CTLs) as described previously (13, 32). SIV-specific CTL frequencies at week 12 were much higher than those at week 2 for all five macaques challenged with mutant SIVs. In contrast, Gag-specific CTL frequencies at week 12 were lower than those at week 2 for four of five animals; the remaining macaque, R06-035, mounted $Gag_{367-381}$-specific CTL responses. Importantly, in all animals challenged with mutant SIVs, SIV-specific CTL

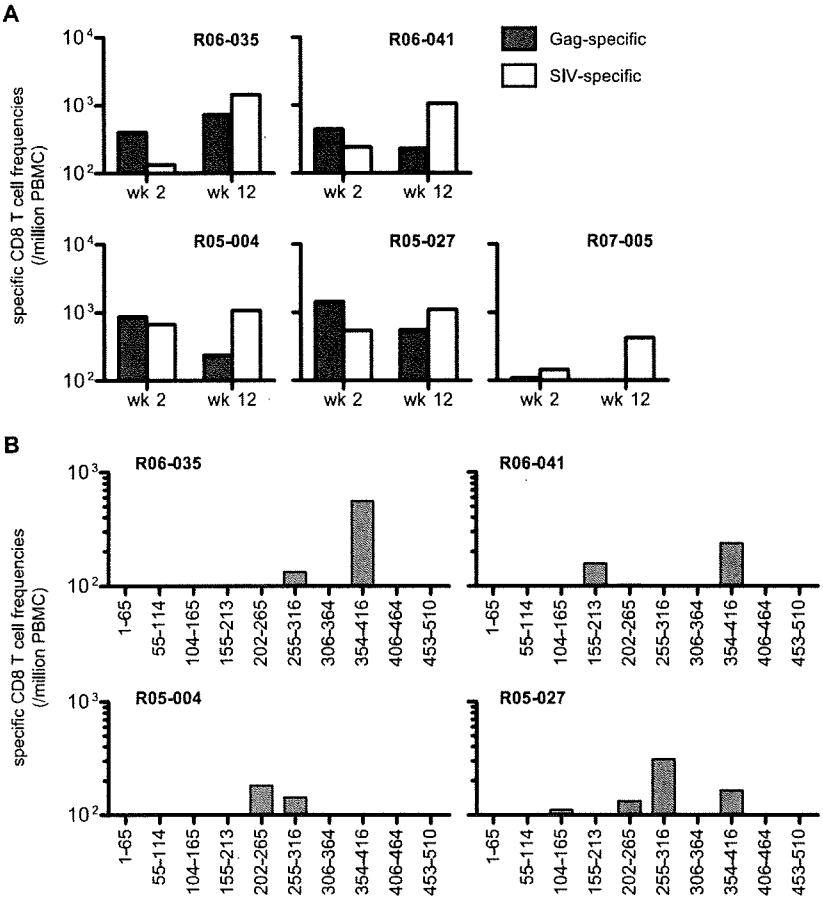


FIG. 5. SIV-specific $CD8^+$ T-cell responses after mutant SIV challenge. (A) Gag-specific (closed boxes) and SIV-specific (open boxes) $CD8^+$ T-cell frequencies at 2 weeks or 12 weeks postchallenge. (B) Frequencies of $CD8^+$ T cells specific for pools of SIV Gag peptides. A panel of 117 overlapping peptides (15 to 17 aa in length and overlapping by 10 to 12 aa) spanning the entire SIV Gag amino acid sequence were divided into the following 10 pools (each consisting of 11 or 12 peptides): pool 1, 1st to 65th aa in SIV Gag; pool 2, 55th to 114th aa; pool 3, 104th to 165th aa; pool 4, 155th to 213th aa; pool 5, 202nd to 265th aa; pool 6, 255th to 316th aa; pool 7, 306th to 364th aa; pool 8, 354th to 416th aa; pool 9, 406th to 464th aa; and pool 10, 453rd to 510th aa. The pools were used for stimulation to detect peptide pool-specific $CD8^+$ T cells.

frequencies were at marginal levels or lower than Gag-specific CTL frequencies at week 2, but the former became higher than the latter at week 12. These results indicate an induction of CTL responses specific for SIV antigens other than Gag in all five macaques after mutant SIV challenge.

At week 12 after mutant SIV challenge, Gag-specific CTL responses were undetectable in macaque R07-005 but were still detected in the other four macaques. We then analyzed Gag-specific CTL responses in these four macaques by using a panel of overlapping peptides spanning the entire SIV Gag amino acid sequence (Fig. 5B). In both SIVmac239Gag216S2 44E-challenged animals, R06-035 and R06-041, exhibiting detectable $Gag_{367-381}$-specific CTL responses (data not shown), CTL responses specific for the peptide mixture corresponding to the 354th to 416th aa in SIV Gag were detected at week 12. In addition, we found $Gag_{255-316}$-specific CTL responses in macaque R06-035 and $Gag_{155-213}$-specific CTL responses in macaque R06-041. SIVmac239Gag216S244E247L312V373T-challenged macaques R05-004 and R05-027 showed responses specific for several Gag peptide mixtures, including $Gag_{202-265}$-specific and $Gag_{255-316}$-specific CTL responses. These results indicate an induction of CTL responses specific for Gag epitopes other than the $Gag_{206-216}$, $Gag_{241-249}$, and $Gag_{373-380}$ epitopes after mutant SIV challenge.

DISCUSSION

In the present study, SIVs carrying multiple *gag* CTL escape mutations showed lower replicative abilities than that of the wild type; nonetheless, the *90-120-Ia*-positive vaccinees were able to contain only the latter. This demonstrates that Gag-specific CTL responses did play a central role in the vaccine-based primary containment of wild-type SIVmac239 replication in *90-120-Ia*-positive macaques.

Elicitation of virus-specific T-cell responses by prophylactic vaccination is believed to be a promising strategy for HIV control (3, 24); whether this approach can actually result in HIV control remains unknown. Recent studies have indicated the possibility of reductions in set point viral loads after SIV challenge by prophylactic vaccination inducing T-cell responses in rhesus macaques (19, 22, 34), yet the immune component crucial for the vaccine-based viral control has not been

determined. No clear evidence for a contribution of vaccine-induced CTLs to this viral control has been forthcoming to date, although virus-specific CTL responses have been implicated in exerting strong suppressive pressure on HIV/SIV infection (9, 22). Indeed, viral replication persists even in the presence of CTL responses in the natural course of infection; it has thus remained unclear whether HIV/SIV replication can be controlled by vaccine-induced CTLs. The evidence from the present study now strongly implicates Gag-specific CTL responses as responsible for vaccine-based primary SIV control. This offers the possibility of Gag-specific CTL-based HIV containment by prophylactic vaccination and provides insight into the development of CTL-based AIDS vaccines.

The containment of SIVmac239 but failure to contain SIV mac239Gag216S244E in the vaccinees documents a crucial role for $Gag_{206-216}$-specific and/or $Gag_{241-249}$-specific CTL responses in vaccine-based SIVmac239 containment. Furthermore, challenge with SIVmac239Gag216S244E247L312V373T, possessing diminished viral fitness compared to SIVmac239Gag216S244E, tended to result in higher viral loads, indicating the involvement of $Gag_{373-380}$-specific CTL responses in viral control, while more complete viral evasion of $Gag_{241-249}$-specific CTL recognition by addition of the GagI247L mutation may also contribute to the difference between SIVmac239Gag216S244E and SIVmac239Gag216S244E247L312V373T challenge. Taken together, we conclude that these two or three epitope-specific CTL responses are crucial for primary SIVmac239 control in *90-120-Ia*-positive vaccinees. Conversely, this study implies that viral evasion of recognition by two dominant epitope-specific CTLs can result in failure of primary viral containment but may not be sufficient for abrogation of vaccine efficacy. Thus, analysis of CTL-based vaccine efficacy against SIVs carrying single or multiple CTL escape mutations could contribute to an evaluation of its potential for controlling the replication of highly diversified HIVs.

Our results suggest that SIV- but non-Gag-specific CTLs became predominant after mutant SIV challenge. Additionally, CTLs recognizing Gag regions other than the $Gag_{206-216}$, $Gag_{241-249}$, and $Gag_{373-380}$ epitopes were detected in most cases. These CTL responses may exert suppressive pressure on viral replication but are considered insufficient for controlling replication of the mutant SIVs with lower viral fitness.

Finally, this study also provides evidence indicating a possible abrogation of CTL-based AIDS vaccine efficacy in viral transmission between MHC-I-matched individuals. Indeed, even the mutant SIVs carrying multiple CTL escape mutations were able to replicate persistently in vivo, despite their diminished replicative ability. Transmission of these viruses can result in persistent viral infection and AIDS progression (30). CTL escape mutations resulting in a loss of viral fitness may revert to the wild-type sequence after transmission into MHC-I-mismatched hosts (4, 8, 9, 16, 18, 20), but such reversion does not occur rapidly; alternatively, some may be retained with additional compensatory mutations (6, 7, 30). Thus, there may be a risk of transmission and accumulation of HIV CTL escape variants even among MHC-I-mismatched individuals, resulting in abrogation of CTL-based AIDS vaccine efficacy in a population.

ACKNOWLEDGMENTS

This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology, by a grant from the Japan Health Sciences Foundation, and by grants from the Ministry of Health, Labor, and Welfare in Japan.

The animal experiments were conducted through the Cooperative Research Program in Tsukuba Primate Research Center, National Institute of Biomedical Innovation, with the help of the Corporation for Production and Research of Laboratory Primates. We thank DNAVEC Corp., H. Igarashi, F. Ono, A. Hiyaoka, K. Oto, Y. Yasutomi, M. Miyazawa, A. Kimura, K. Mori, N. Yamamoto, T. Kurata, Y. Nagai, and A. Nomoto for their help.

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Vaccine 26 (2008) 6839–6843

Contents lists available at ScienceDirect

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Evaluation of the immunogenicity of replication-competent V-knocked-out and replication-defective F-deleted Sendai virus vector-based vaccines in macaques

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ARTICLE INFO

Article history:
Received 13 June 2008
Received in revised form
14 September 2008
Accepted 27 September 2008
Available online 16 October 2008

Keywords:
AIDS vaccine
Sendai virus vector
T-cell response

ABSTRACT

Viral vectors are promising vaccine tools for eliciting antigen-specific T-cell responses. We previously showed the potential of recombinant Sendai virus (SeV) vectors to induce virus-specific T-cell responses in macaque AIDS models. Here, we have evaluated the immunogenicity of replication-competent V-knocked-out and replication-defective F-deleted SeV vectors in macaques. Intranasal replication-competent and replication-defective SeV immunizations both elicited robust systemic antigen-specific T-cell responses, whereas the responses induced by the former were more durable than those by the latter. However, even the latter-induced T-cell responses remained detectable in a local, retropharyngeal lymph node two months after the immunization. These findings are useful for establishment of a vaccine protocol using SeV vectors.

1. Introduction

Virus-specific T-cell responses play an important role in the control of human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV) infection [1–7]. Especially, virus-specific $CD8^+$ cytotoxic T lymphocyte (CTL) responses exert suppressive pressure on HIV-1 and SIV replication. Hence, efficient T-cell induction is a key strategy for AIDS vaccine development, and recombinant viral vectors are promising tools for its elicitation [8].

We previously developed an AIDS vaccine using a recombinant Sendai virus (SeV) vector and showed the potential of this vector for efficient CTL induction in macaques [9–11]. SeV, murine parainfluenza virus type 1 (PIV-1), is an enveloped virus with a negative-sense RNA genome. Its natural host is mice and it is considered nonpathogenic for primates including humans [12]. Indeed, our analysis showed no disease progression by SeV infection of macaques or no detectable SeV transmission from SeV-infected to uninfected macaques [10]. A clinical phase I trial of SeV as a vaccine against human PIV-1 indicated its safety in humans [13].

We have two types of SeV vectors expressing SIV Gag, a replication-competent V-knocked-out V(-)SeV-Gag and a replication-defective F-deleted F(-)SeV-Gag [9,10,14–18]. The former V(-)SeV vector carrying a genome whose V gene, an SeV accessory gene, is knocked out is transmissible; i.e., infectious V(-)SeV virions can be produced from V(-)SeV-infected cells and transmitted to another cells. This V(-)SeV has attenuated virulence in mice but keeps the ability to induce efficient gene transfer [15,19]. In contrast, the latter F(-)SeV vector is non-transmissible; i.e., F(-)SeV-infected cells are unable to produce infectious, transmissible F(-)SeV virions [17]. This F(-)SeV vector also maintains efficient transduction ability with a high level of transgene expression. Therefore, the non-transmissible F(-)SeV vector, which may be advantageous in safety issues, is a promising delivery tool for gene therapy and vaccination in humans.

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doi:10.1016/j.vaccine.2008.09.074

In our previous studies [9,11,18], V(-)SeV-Gag and F(-)SeV-Gag immunizations both elicited Gag-specific T-cell responses in macaques, but we have not precisely compared their abilities to induce T-cell responses. In the present study, to evaluate their immunogenicity, we have examined systemic and local antigen-specific T-cell responses after immunization with replication-competent V(-)SeV-Gag or replication-defective F(-)SeV-Gag in macaques.

2. Materials and methods

2.1. Animals

Cynomolgus macaques (*Macaca fascicularis*) and rhesus macaques (*Macaca mulatta*) were maintained in accordance with the guidelines for laboratory animals of the National Institute of Infectious Diseases and the National Institute of Biomedical Innovation. Blood collection, lymph node (LN) biopsy, and vaccination were performed under ketamine anesthesia. Animals received a DNA vaccine followed by a single boost with V(-)SeV-Gag or F(-)SeV-Gag as described previously [11]. The DNA, CMV-SHIVdEN, used for the vaccination was constructed from an *env*- and *nef*-deleted $SHIV_{MD14YE}$ molecular clone DNA [20] and has the genes encoding SIVmac239 Gag, Pol, Vif, and Vpx, SIVmac239-HIV-1 chimeric Vpr, and HIV-1 Tat and Rev [9,11]. At the DNA vaccination, animals received 5 mg of CMV-SHIVdEN DNA intramuscularly. Six weeks after the DNA prime, animals intranasally received a single boost with 1×10^8 cell infectious units (CIU) of replication-competent V(-)SeV-Gag or 6×10^9 CIU of replication-defective F-deleted F(-)SeV-Gag [9,10,18].

2.2. Measurement of virus-specific T-cell responses

We measured virus-specific T-cell levels by flow-cytometric analysis of interferon-γ (IFN-γ) induction after specific stimulation as described previously [11,21]. In brief, peripheral blood mononuclear cells (PBMCs) were cocultured with autologous herpesvirus papio-immortalized B lymphoblastoid cell lines infected with a vaccinia virus vector expressing SIVmac239 Gag for Gag-specific stimulation or a SeV for SeV-specific stimulation. Intracellular IFN-γ staining was performed using CytofixCytoperm kit (Becton Dickinson, Tokyo, Japan). Fluorescein isothiocyanate (FITC)-conjugated anti-human CD4, peridinin chlorophyll protein (PerCP)-conjugated anti-human CD8, allophycocyanin (APC)-conjugated anti-human CD3, and phycoerythrin (PE)-conjugated anti-human IFN-γ antibodies (Becton Dickinson, Tokyo, Japan) were used. Specific T-cell levels were calculated by subtracting nonspecific IFN-γ^+ T-cell frequencies from those after Gag-specific or SeV-specific stimulation. Specific T-cell frequencies less than 100 cells per million PBMCs were considered negative, those between 100 and 200 borderline, and those greater than 200 positive.

2.3. Statistical analysis

Statistical analysis was performed by Prism software version 4.03 with significance levels set at $p < 0.05$ (GraphPad Software, Inc., San Diego, CA). Gag-specific $CD8^+$ T-cell frequencies were log-transformed and compared between the two groups, V(-)SeV-Gag-vaccinated and F(-)SeV-Gag-vaccinated, by unpaired two-tailed *t* test.

3. Results

3.1. Both replication-competent V(-)SeV-Gag and replication-defective F(-)SeV-Gag elicited robust Gag-specific T-cell responses

Four cynomolgus macaques received a DNA vaccine followed by a boost with replication-competent V(-)SeV-Gag, while seven macaques were boosted with replication-defective F(-)SeV-Gag after the DNA prime. We then examined Gag-specific T-cell responses in these macaques by flow-cytometric analysis of IFN-γ induction after specific stimulation. All four V(-)SeV-Gag-boosted and all seven F(-)SeV-Gag-boosted macaques showed efficient induction of Gag-specific T-cell responses after the boost (Fig. 1A). While Gag-specific $CD4^+$ T-cell responses were predominant in some animals such as macaque C97-018, Gag-specific $CD8^+$ T-cell responses were detectable in all macaques one week after the boost (Fig. 1A), although these responses were undetectable before the boost (data not shown). There was no significant difference in Gag-specific $CD4^+$ T-cell or $CD8^+$ T-cell levels one week after the boost between the V(-)SeV-Gag-boosted and the F(-)SeV-Gag-boosted macaques ($p = 0.3779$ [CD4] and $p = 0.7963$ [CD8] by unpaired two-tailed *t* test). We also examined SeV-specific T-cell responses and found efficient induction of SeV-specific T-cell responses in

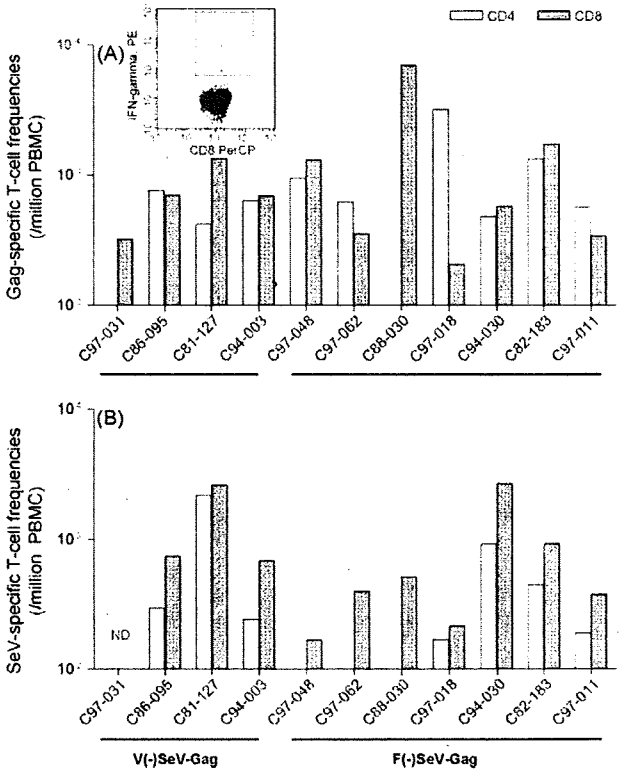

Fig. 1. Antigen-specific T-cell frequencies in PBMCs one week after SeV boost. (A) Gag-specific $CD4^+$ T-cell (open boxes) and $CD8^+$ T-cell (closed boxes) frequencies one week after an intranasal boost with V(-)SeV-Gag (macaques C97-031, C86-095, C81-127, and C94-003) or F(-)SeV-Gag (macaques C97-048, C97-062, C88-030, C97-018, C94-030, C82-183, and C97-011). A representative dot plot gated on $CD3^+$ $CD8^+$ lymphocytes (macaque C86-095) after Gag-specific stimulation is shown at the top. Geometric means of Gag-specific $CD4^+$ T-cell and $CD8^+$ T-cell frequencies were 3.8×10^2 cells/million PBMCs and 6.7×10^2 cells/million PBMCs in the V(-)SeV-Gag-boosted group and 6.8×10^2 cells/million PBMCs and 8.1×10^2 cells/million PBMCs in the F(-)SeV-Gag-boosted group. (B) SeV-specific $CD4^+$ T-cell (open boxes) and $CD8^+$ T-cell (closed boxes) frequencies one week after a boost with V(-)SeV-Gag or F(-)SeV-Gag. ND, not determined.

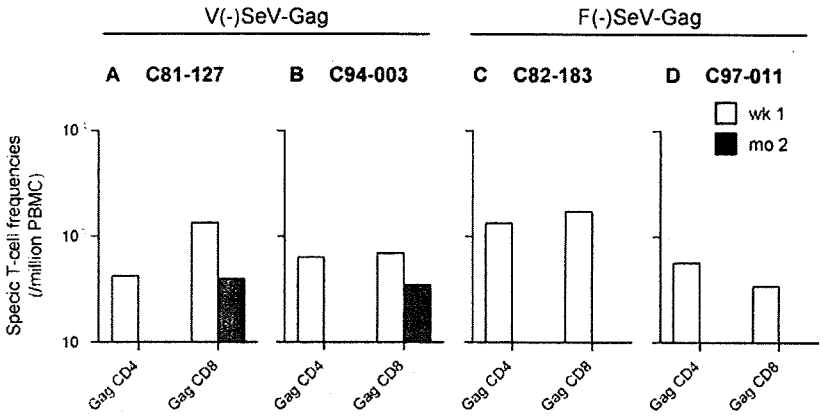

Fig. 2. Gag-specific T-cell frequencies in PBMCs two months after SeV boost. Gag-specific CD4⁺ T-cell and CD8⁺ T-cell frequencies one week (open boxes) or two months (closed boxes) after an intranasal boost with V(-)SeV-Gag (macaques C81-127 (A) and C94-003 (B)) or F(-)SeV-Gag (macaques C82-183 (C) and C97-011 (D)) are shown.

all (Fig. 1B). Thus, replication-competent V(-)SeV-Gag boost and replication-defective F(-)SeV-Gag boost both elicited robust Gag-specific and SeV-specific T-cell responses.

3.2. Replication-competent V(-)SeV-Gag elicited more durable Gag-specific T-cell responses than replication-defective F(-)SeV-Gag

We followed up two of the V(-)SeV-Gag-boosted macaques and two of the F(-)SeV-Gag-boosted macaques, and examined Gag-specific T-cell responses in these macaques two months after the boost. Gag-specific CD4⁺ T-cell responses became undetectable in both groups. However, Gag-specific CD8⁺ T-cell responses were still detectable in both of the V(-)SeV-Gag-boosted macaques but became undetectable in both of the F(-)SeV-Gag-boosted macaques (Fig. 2).

We then combined this result with our previous data obtained from four V(-)SeV-Gag-boosted macaques and four F(-)SeV-Gag-boosted macaques [11], and compared Gag-specific CD8⁺ T-cell levels between the V(-)SeV-Gag-boosted and the F(-)SeV-Gag-boosted groups by statistical analysis (Fig. 3). No significant difference in Gag-specific CD8⁺ T-cell levels one week after the boost was observed between the two groups ($p = 0.5112$ by unpaired two-tailed t test). However, a few months after the boost, the V(-)SeV-Gag-boosted macaques showed significantly higher levels of Gag-specific CD8⁺ T-cell responses than the F(-)SeV-Gag-boosted ($p = 0.0169$ by unpaired two-tailed t test). Indeed, Gag-specific CD8⁺ T-cell responses were still detectable in all six V(-)SeV-Gag-boosted macaques but became undetectable in five of six F(-)SeV-Gag-boosted macaques. These results indicate that the V(-)SeV-Gag boost elicited more durable Gag-specific CD8⁺ T-cell responses compared to the F(-)SeV-Gag boost.

3.3. Gag-specific T-cell responses were maintained in the lymph nodes around the nasal mucosa

The V(-)SeV-Gag-boosted and F(-)SeV-Gag-boosted macaques in the present study were euthanized one week, two weeks, or two months after the boost. We then obtained the axillary LN and the retropharyngeal LN by autopsy from two of four V(-)SeV-Gag-boosted and all seven F(-)SeV-Gag-boosted macaques. We examined Gag-specific CD8⁺ T-cell frequencies in these LNs (Fig. 4), although we failed to obtain autopsy samples from V(-)SeV-Gag-boosted C86-095 and C94-003. The retropharyngeal LN is known to receive the primary lymphocyte drainage from the nasal cavity [22]. In all animals, Gag-specific CD8⁺ T-cell frequencies in the retropharyngeal LNs were no less than those in the axillary LNs. Both V(-)SeV-Gag-boosted animals showed high levels of Gag-specific CD8⁺ T-cell responses. Remarkably, even in F(-)SeV-Gag-boosted macaques C82-183 and C97-011 that were euthanized two months after the boost, Gag-specific CD8⁺ T-cell responses were clearly

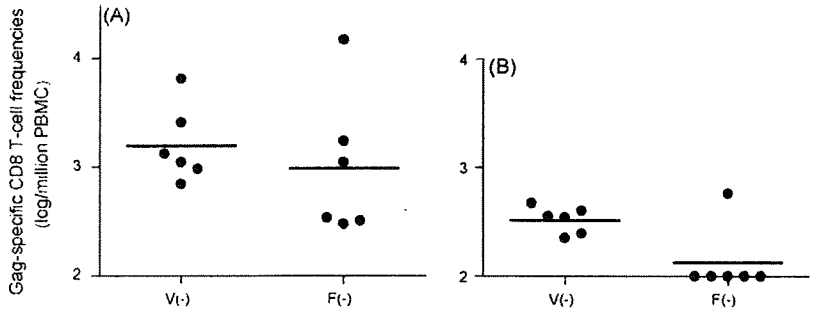

Fig. 3. Comparison of Gag-specific CD8⁺ T-cell frequencies between the V(-)SeV-Gag-boosted and the F(-)SeV-Gag-boosted macaques. The replication-competent V(-)SeV-Gag-boosted group (V[-], $n = 6$) is consisting of macaques C81-127 and C94-003 and previously-reported macaques V1, V2, V3, and V4 [11]. The replication-defective F(-)SeV-Gag-boosted group (F[-], $n = 6$) is consisting of macaques C82-183 and C97-011 and previously-reported macaques V5, V6, V7, and V8 [11]. Gag-specific CD8⁺ T-cell frequencies one week (A) or two or three months (B) after the boost were log-transformed and compared between the two groups by unpaired two-tailed t test. Geometric means of Gag-specific CD8⁺ T-cell frequencies one week after the boost were 1.6×10^3 cells/million PBMCs in the V(-)SeV-Gag-boosted group and 1.0×10^3 cells/million PBMCs in the F(-)SeV-Gag-boosted group. Geometric means of Gag-specific CD8⁺ T-cell frequencies two or three months after the boost were 3.3×10^2 cells/million PBMCs in the V(-)SeV-Gag-boosted group and 1.3×10^2 cells/million PBMCs in the F(-)SeV-Gag-boosted group.

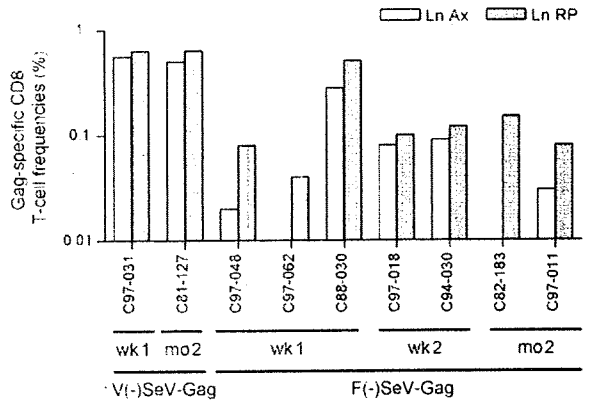

Fig. 4. Gag-specific CD8⁺ T-cell frequencies in LNs after SeV boost. Macaques were euthanized one week (macaques C97-031, C97-048, C97-062, and C88-030), two weeks (macaques C97-018 and C94-030), or two months (macaques C81-127, C82-183 and C97-011) after boost. Gag-specific CD8⁺ T-cell frequencies (%) in CD8⁺ T lymphocytes in axillary LNs (Ln Ax, open boxes) and retropharyngeal LNs (Ln RP, closed boxes) obtained at autopsy are shown.

detected in the retropharyngeal LNs although not in the axillary LNs.

4. Discussion

Recombinant viral vectors are promising vaccine tools for eliciting antigen-specific T-cell responses. Some of them are derived from nonpathogenic parental viruses but others are from pathogenic ones. For their clinical uses in safe, especially in the latter cases, viral vectors are prepared from attenuated viruses but not from pathogenic ones themselves. Alternatively, viral vectors are constructed by deleting or revising viral genomes for attenuation; some are constructed to lose their replication competency without losing their ability to express antigens.

SeV is pathogenic for mice but is considered nonpathogenic for primates including humans [10,12,13]. Thus, replication-competent SeV vectors including V(-)SeV may be used for humans, but we also have a replication-defective F(-)SeV vector that has the ability to efficiently express antigens. We previously showed that both V(-)SeV-Gag and F(-)SeV-Gag vector vaccines can elicit Gag-specific T-cell responses in macaques [9,11,18], but their immunogenicity has not been evaluated precisely. Indeed, our previous analyses showed that infectious V(-)SeV in the nasal swab became undetectable in a week after its intranasal inoculation, indicating that V(-)SeV replication is not so efficient in macaques [10]. Thus, it has remained unclear whether this short period of SeV replication can significantly affect T-cell responses.

The present study showed that both V(-)SeV-Gag boost and F(-)SeV-Gag boost elicited robust systemic Gag-specific T-cell responses, whereas the responses induced by the former were more durable than those by the latter. Indeed, systemic Gag-specific CD8⁺ T-cell responses were maintained detectable in all the V(-)SeV-Gag-boosted macaques but became undetectable in most of the F(-)SeV-Gag-boosted macaques in a few months. This durability of Gag-specific CD8⁺ T-cell responses may be due to the short period of V(-)SeV-Gag replication. Both groups showed similar levels of Gag-specific and SeV-specific CD4⁺ T-cell responses, and we found no evidence indicating involvement of CD4⁺ T-cell responses in induction or maintenance of Gag-specific CD8⁺ T-cell responses. Interestingly, our results suggested that, even after a few months after the F(-)SeV-Gag boost, localized Gag-specific CD8⁺ T-cell responses were maintained detectable in the retropharyngeal LNs around the nasal mucosa despite the absence of detectable systemic Gag-specific CD8⁺ T-cell responses. We obtained the tonsils at autopsy from five F(-)SeV-Gag-boosted macaques and found efficient Gag-specific CD8⁺ T-cell responses in four of them (C97-062: 0.02%; C88-030: 0.74%; C97-018: 0.25%, C94-030: 0.22%, and C97-011: 0.12%). These results imply possible Gag-specific CD8⁺ T-cell responses in mucosal tissues by intranasal SeV-Gag immunization, which may be effective against HIV-1/SIV transmission.

In development of a prophylactic T cell-based AIDS vaccine, what to be induced by vaccination is not effector but memory T cells that can efficiently respond to viral exposure. In the previous experiment of SIVmac239 challenge three months after boost [11], we found no significant difference in protective efficacy between V(-)SeV-Gag-boosted and F(-)SeV-Gag-boosted macaques; two of four V(-)SeV-Gag-boosted and three of four F(-)SeV-Gag-boosted macaques controlled SIV replication. Even the F(-)SeV-Gag-boosted macaques that had no detectable Gag-specific CD8⁺ T cells at SIV challenge showed rapid secondary responses and controlled SIV replication. The present study suggested a possibility that Gag-specific CD8⁺ T-cell responses induced by F(-)SeV-Gag boost can be maintained in a local, retropharyngeal LN, and these localized, persistent Gag-specific CD8⁺ T-cell responses may contribute to the rapid secondary responses after SIV challenge. Thus, replication-defective F(-)SeV-Gag-induced Gag-specific CD8⁺ T-cell responses that are less durable compared to those induced by replication-competent V(-)SeV-Gag may be sufficient for SIV control.

In summary, we showed that both replication-competent V(-)SeV-Gag and replication-defective F(-)SeV-Gag vectors have the potential to elicit robust Gag-specific CD8⁺ T-cell responses in macaques, whereas the responses induced by the former are more durable than those by the latter in this case. However, our results indicated that even the latter-induced Gag-specific CD8⁺ T-cell responses can persist in local LNs around the nasal mucosa, suggesting a possibility of HIV-1/SIV control even in the absence of detectable vaccine-induced persistent virus-specific CD8⁺ T-cell responses at the virus exposure. Although the vaccine-induced virus-specific CD8⁺ T-cell levels that are sufficient or adequate for HIV-1/SIV control remain unclear, these findings provide important data for establishment of a vaccine protocol using SeV vectors.

Acknowledgements

This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology and grants from the Ministry of Health, Labor, and Welfare in Japan.

The animal experiments were conducted through the Cooperative Research Program in Tsukuba Primate Research Center, National Institute of Biomedical Innovation with the help of the Corporation for Production and Research of Laboratory Primates. We thank A. Kato, M. Kano, H. Nakamura, F. Ono, A. Hiyaoka, K. Oto, K. Komatsuzaki, H. Ogawa, H. Akari, Y. Yasutomi, N. Yamamoto, T. Kurata, A. Nomoto, and Y. Nagai for their help.

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PRIORITY PAPER EVALUATION

Genetic factors that confer sensitivity to HAART in HIV-infected subjects: implication of a benefit of an earlier initiation of HAART

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Evaluation of: Ahuja SK, Kulkarni H, Catano G *et al.*: *CCL3L1-CCR5* genotype influences durability of immune recovery during antiretroviral therapy of HIV-1-infected individuals. *Nat. Med.* 14(4), 413–420 (2008). It is widely accepted that the effect of highly active antiretroviral therapy (HAART) varies widely among HIV-infected individuals. Host genetic factors are thought to be linked to the sensitivity to HAART in HIV-infected individuals. Ahuja *et al.* attempted to identify the genes that determine the sensitivity to HAART in HIV-infected subjects. Based on the hypothesis that CD4$^+$ depletion and the recovery process in HIV-infected subjects are under the control of specific common genetic pathways, they evaluated the associations of genetic variations, such as *CCR5* genotype, *CCL3L1* copy number variation and *HLA* alleles, with the sensitivity to HAART in two cohorts from the USA. They found that the *CCL3L1-CCR5* genetic risk status, but not *HLA-B*57*, is apparently a good predictor of the recovery rate of CD4$^+$ T cells during HAART. In particular, the recovery rate of CD4$^+$ T cells during HAART has the most sensitive association with the copy number of *CCL3L1*. Furthermore, Ahuja *et al.* studied the impact of *CCL3L1-CCR5* genetic risks in HIV-infected individuals initiating HAART during acute or early infection. They suggested that *CCL3L1-CCR5* genetic risk status may be a useful guide in deciding whether to initiate HAART in HIV-infected subjects with a level of 350 CD4$^+$ T cells/mm^3 or more. This study has provided a critical breakthrough in predicting the response to HAART in HIV-infected subjects.

The combination of antiretroviral drugs known as highly active antiretroviral therapy (HAART) has drastically improved the prognosis for great numbers of HIV-infected patients in a very short period of time. Ahuja *et al.* have identified a new genetic mechanism controlling susceptibility to HAART [1]. The AIDS death rate in the USA declined by more than two-thirds within 2 years of the appearance of the protease inhibitors [2]. However, it is widely reported that the effect of HAART, such as the level of viral replication suppression and the recovery rate of CD4$^+$ T-cell counts, varies widely among HIV-infected individuals. Host genetic factors in all likelihood are linked to the sensitivity to HAART in HIV-infected individuals. In fact, there have been several studies performed to evaluate the impact of genetic factors on the sensitivity to HAART. The main features and outcomes of these studies are summarized in Table 1. The majority of these studies have been focused on sequence variations in *HLA-B* [3,4] and *CCR5* [5–9].

Ahuja *et al.* sought to identify the genes that determine the sensitivity to HAART in chronic HIV-infected subjects. They hypothesized that CD4$^+$ depletion and the recovery process are under the control of specific common genetic pathways, and that genetic factors determining HIV-1/AIDS susceptibility are tightly linked to the sensitivity to HAART. In the study, they focused on three genetic variations, *CCR5* genotypes, *CCL3L1* copy number variations and *HLA-B*57*. These three genes have been reported to be tightly linked to HIV/AIDS susceptibility. Sequence variations in *CCR5*, which result in reduced or absent cell-surface expression of the HIV coreceptor CCR5, decrease the susceptibility to HIV-1 infection [10–13]. CCL3L1, a natural ligand for CCR5, is a potent HIV-1-suppressive chemokine. *CCL3L1* copy number variation is tightly linked to HIV/AIDS susceptibility, and a lower copy number is associated with both an enhanced risk for acquiring HIV-1 and also progressing more rapidly to AIDS and death [14,15]. *HLA-B*57* has also been reported to be associated with a better prognosis in HIV-infected individuals [10,11,16,17].

Furthermore, the report has shed new light on the benefits of an earlier initiation of HAART. In the current guidelines for the starting of HAART [101], the earlier initiation of HAART, especially in HIV-infected individuals with a level of 350 CD4$^+$ T cells/mm^3 or more, remain controversial. The authors studied the impacts of

Keywords: CCL3L1, CCR5, HAART, HIV-1, HLA

10.2217/14622416.9.9.1347 © 2008 Future Medicine Ltd ISSN 1462-2416

Table 1. The main features and outcomes of seven studies performed to evaluate the effects of HLA genotypes and *CCR5Δ32* on the clinical course after HAART.

Gene	Study	Sample size	Cohort and ethnic backgrounds	Effects on the clinical course after HAART	Ref.
HLA-B	Brumme *et al.* (2007)	n = 765	HOMER cohort, British Colombia, Canada	Impaired CD4+ T-cell recovery in HLA class I homozygosity Slower virologic suppression in subjects with uncommon HLA alleles	[3]
	Rauch *et al.* (2008)	n = 265	Swiss HIV cohort study and Western Australian HIV cohort study	Impaired CD4+ T-cell recovery in HLA-Bw4 homozygosity	[4]
CCR5Δ32	Bratt *et al.* (1998)	n = 147	Subjects from Sweden	No significant effect on the efficacy of HAART	[5]
	Valdez *et al.* (1999)	n = 113	White subjects from Cleveland, OH, USA	A better response (viral load and CD4+ T cells) in CCR5 wt/Δ32 heterozygotes in comparison with CCR5 wt/wt homozygotes	[6]
	O'Brien *et al.* (2000)	n = 273	AIDS Clinical Trial Group 343 study, Caucasians with CD4+ T-cell ≥200/mm^3 and plasma HIV RNA ≥1000 copies/ml	No significant effect on the efficacy of HAART	[7]
	Kasten *et al.* (2000)	n = 107	Subjects from Germany	A better response (viral load and CD4+ T cells) in CCR5 wt/Δ32 heterozygotes in comparison with CCR5 wt/wt homozygotes	[8]
	Laurichesse *et al.* (2007)	n = 565	French Agence Nationale de Recherrche SERCO/HEMOCO cohort	A better virological response in CCR5 wt/Δ32 heterozygotes in comparison with CCR5 wt/wt homozygotes No significant effect on survival or AIDS-free survival	[9]

HAART: Highly active antiretroviral therapy; HEMOCO: Hemophiliacs HIV-1 infection; HLA: Human leukocyte antigen; HOMER: HAART Observational Medical Evaluation and Research SERCO: Seroconversion; wt: Wild-type.

CCL3L1-CCR5 genetic risks in HIV-infected individuals initiating HAART during acute or early infection.

Genes responsible for HAART sensitivity in chronic HIV-infected subjects

In the study by Ahuja *et al.*, the Wilford Hall Medical Center (WHMC; TX, USA) cohort was studied to evaluate the associations of genetic variations with the sensitivity to HAART in chronic HIV-infected subjects. The WHMC cohort is a component of the USA Military Tri-Service AIDS Clinical Consortium Natural History Study, which is one of the largest cohorts of HIV-positive patients followed prospectively at a single medical center. In the WHMC cohort, 502 HIV-infected subjects received HAART.

Ahuja *et al.* classified a large cohort of HIV-1-infected subjects into three *CCL3L1-CCR5* genetic risk groups on the basis of the copy number of *CCL3L1* and *CCR5* genotype [11]. The high-risk group possessed both a population-specific low *CCL3L1* copy number (*CCL3L1^{low}*) and detrimental *CCR5* variations (*CCR5^{det}*). The low-risk group had both a population-specific high *CCL3L1* copy number (*CCL3L1^{high}*) and non-detrimental *CCR5* variations (*CCR5^{nondet}*). The moderate risk group harbored either one or the other of the two risk factors, *CCL3L1^{low} or CCR5^{det}*. Their cohort was also categorized based on the levels of viral load suppression after HAART to investigate whether, among subjects with a similar viral load suppression, the rate and extent of CD4+ T-cell recovery differed according to the *CCL3L1-CCR5* genetic risk status. They also categorized the cohort based on the levels of CD4+ T cells with which HAART was started. They found that the *CCL3L1-CCR5* genetic risk status is apparently a good predictor of the recovery rate of CD4+ T cells during HAART, especially in those who attained viral load suppression after HAART and those who started

HAART with levels of less than 350 $CD4^+$ T cells/mm^3. They also reported that the recovery rate of $CD4^+$ T cells during HAART has the most sensitive association with the copy number of *CCL3L1*.

On the other hand, possession of *HLA-B*57* had no impact on the recovery rate of $CD4^+$ T cells in HIV-infected subjects receiving HAART. Surprisingly, individuals possessing *HLA-B*57*, which was associated with a delayed disease progress, had an impaired $CD4^+$ T-cell recovery during the first 2–3 years after starting HAART. Similar findings have also been reported by others. Rauch *et al.* have reported that *HLA-Bw4* homozygosity, which has been reported to be linked to a better prognosis in HIV-infected subjects, was associated with an impaired $CD4^+$ T-cell recovery after HAART [4]. They also reported that the possession of *HLA-B*57* was likely to be associated with an impaired $CD4^+$ T-cell recovery. The basis for *HLA-B*57* protection seems to be related, in part, to a highly conserved immunodominant epitope in Gag, a response to which seems to confer early protection during acute infection [18]. HIV-1 specific $CD8^+$ T-cell response restricted by *HLA-B*57* provides a potential mechanism for epidemiological protection, even though it might have no beneficial effects on $CD4^+$ T-cell recovery during HAART.

Interestingly, *HLA-B*57* has been reported to be associated with a hypersensitivity reaction to adacavir, a nucleotide reverse-transcriptase inhibitor [19,20]. The current treatment guidelines recommend screening for *HLA-B*5701* before starting patients on an abacavir-containing regimen, to reduce the risk of a hypersensitivity reaction [101]. *HLA-B*57*-related mechanisms for HAART resistance and hypersensitivity to abacavir are not clear; however, we must pay extra attention to HIV-infected subjects possessing *HLA-B*57* during the course of HIV treatment.

Genetic risk status & earlier initiation of HAART in acute or early HIV-infected subjects

Despite the possible benefits of HAART in HIV-infected subjects with $CD4^+$ T-cell counts over 350 cells/mm^3, there are a couple of reasons which mitigate against the earlier initiation of HAART [101]. However, the earlier initiation of HAART in HIV-infected subjects with a high risk of disease progression appears to be indicated.

Ahuja *et al.* studied the Acute Infection and Early Disease Research Program (AIEDRP) cohort to evaluate the impacts of *CCL3L1-CCR5* genetic risks on the sensitivity to HAART in HIV-infected individuals initiating HAART during acute or early infection [1]. In this prospective study, 315 HIV-infected subjects with signs or symptoms of an acute retroviral syndrome or evidence of recent HIV infection were enrolled. Among the subjects who received HAART during acute infection and who attained viral load suppression, a low genetic risk of *CCL3L1-CCR5* was associated with a greater recovery rate of $CD4^+$ T cells in comparison with a moderate or high genetic risk. They suggested that *CCL3L1-CCR5* genetic risk status may be a useful guide in deciding whether to initiate HAART in HIV-infected subjects with a level of 350 $CD4^+$ T cells/mm^3 or more.

Future perspective

Ahuja *et al.* provided critically important information regarding the genes that determine the sensitivity to HAART in HIV-infected subjects. However, further studies are required to assess the following four issues. First, the association between genetic variations and the HAART sensitivity should be replicated by using other population samples. The authors evaluated two cohorts, WHMC and AIEDRP, both of which were from the USA. Since subtype B of HIV-1 is the most prevalent in the USA [2], it remains possible that the genotype–phenotype interactions observed in this study might not be identified in the context of other HIV-1 subtypes.

Second, additional genes might be linked to the HAART sensitivity in HIV-infected subjects. Other HIV/AIDS susceptible genes [10,11,15] and/or genes linked to drug metabolism might be good candidates. Recently, genome-wide association studies have proven to be a powerful approach to identify the genes responsible for human common diseases [21,22]. Scanning the entire human genome by a genome-wide association study could serve an alternative approach to identifying the genes tightly linked to the HAART sensitivity in HIV-infected subjects.

Third, what is the mechanism by which the *CCL3L1-CCR5* genetic risk status influences the sensitivity to HAART? The HIV-1 entry-dependent effect through the interaction among HIV-1, *CCR5* and *CCL3L1* has been widely accepted as a key mechanism of inhibiting HIV-1 infection [10,11], because CCR5 is a HIV co-receptor and *CCL3L1* is a natural ligand of *CCR5*. Alternatively, viral entry-independent mechanisms have been suggested as the major protective

mechanisms acting through the *CCL3L1* and *CCR5* axis in their previous study [23]. These two mechanisms might have synergistic and/or additive effects with HAART-induced viral load suppression. However, the HIV-1 entry-independent mechanisms remain to be elucidated. Shalekoff *et al.* reported that *CCL3L1* copy numbers were tightly linked to $CD4^+$ and $CD8^+$ T-cell responses to the HIV-1 Gag protein [24]. *CCL3L1-CCR5* genetic risk status might influence cell-mediated immunity such as HIV-1-specific $CD4^+$ and $CD8^+$ T-cell responses. Understanding the HIV-1 entry-independent mechanisms would be useful for establishing new strategies of HIV treatment and to develop new types of HIV drugs.

Finally, the long-term effect of earlier initiation of HAART on survival and/or AIDS-free survival should be evaluated. Several studies have demonstrated that an earlier initiation of HAART is associated with a greater recovery rate of $CD4^+$ T cells, but not with a significant reduction in the risk of non-AIDS events/morbidity [101]. Long-term and prospective studies in HIV-infected subjects with high genetic risk are needed to definitively evaluate the benefit of the earlier initiation of HAART.

It is widely accepted that genetic variations are tightly linked to the sensitivity to HAART in HIV-infected subjects. Ahuja *et al.* provided a critical breakthrough to predict the response to HAART in HIV-infected subjects. They opened the door of 'personalized medicine' in HIV therapy. It is to be hoped that further progress will provide great insights into an understanding of the contribution of genetic factors to the response to HIV treatment.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Executive summary

Study design

- The Wilford Hall Medical Center (TX, USA) cohort was studied to evaluate the associations of genetic variations with the sensitivity to highly active antiretroviral therapy (HAART) in chronic HIV-infected subjects.
- The Acute Infection and Early Disease Research Program cohort was studied to evaluate the impacts of genetic risks on the sensitivity to HAART in HIV-infected subjects initiating HAART during acute or early HIV infection.

Analyses performed

- The associations of *CCR5* genotypes, *CCL3L1* copy number variations and *HLA* alleles with HAART sensitivity (viral load suppression and $CD4^+$ T-cell recovery rate) were evaluated.
- HIV-1 infected subjects were classified into three *CCL3L1-CCR5* genetic risk groups – low, moderate and high – on the basis of the copy number of *CCL3L1*, as well as *CCR5* genotype.

Results

- *CCL3L1-CCR5* genetic risk status, but not *HLA-B*57*, were associated with the recovery rate of $CD4^+$ T cells during HAART.
- The recovery rate of $CD4^+$ T cells during HAART is the most sensitive activity associated with the copy number of *CCL3L1*.
- Among the subjects who received HAART during acute infection and who attained viral load suppression, a low genetic risk of *CCL3L1-CCR5* was associated with a greater recovery rate of $CD4^+$ T cells in comparison with a moderate or high genetic risk.

Conclusion

- *CCL3L1-CCR5* genetic risk status, but not *HLA-B*57*, is apparently a good predictor of the recovery rate of $CD4^+$ T cells during HAART.
- *CCL3L1-CCR5* genetic risk status may be a useful guide in deciding whether to initiate HAART during acute or early HIV infection.

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Immunogenetics
DOI 10.1007/s00251-008-0332-0

ORIGINAL PAPER

Natural selection in the *TLR*-related genes in the course of primate evolution

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Received: 4 June 2008 / Accepted: 4 September 2008

Abstract The innate immune system constitutes the front line of host defense against pathogens. Toll-like receptors (TLRs) recognize molecules derived from pathogens and play crucial roles in the innate immune system. Here, we provide evidence that the TLR-related genes have come under natural selection pressure in the course of primate evolution. We compared the nucleotide sequences of 16 TLR-related genes, including *TLRs* (*TLR1–10*), *MYD88*, *TILAP*, *TICAM1*, *TICAM2*, *MD2*, and *CD14*, among seven primate species. Analysis of the non-synonymous/synonymous substitution ratio revealed the presence of both strictly conserved and rapidly evolving regions in the TLR-related genes. The genomic segments encoding the intracellular Toll/interleukin 1 receptor domains, which exhibited lower rates of non-synonymous substitution, have undergone purifying selection. In contrast, *TLR4*, which carried a high proportion of non-synonymous substitutions in the part of extracellular domain spanning 200 amino acids, was found to have been the suggestive target of positive Darwinian selection in primate evolution. However, sequence analyses from 25 primate species, including eight hominoids, six Old World monkeys, eight New World monkeys, and three prosimians, showed no evidence that the pressure of positive Darwinian selection has shaped the pattern of sequence variations in *TLR4* among New World monkeys and prosimians.

Keywords Toll-like receptor · Natural selection · Primate evolution

Electronic supplementary material The online version of this article (doi:10.1007/s00251-008-0332-0) contains supplementary material, which is available to authorized users.

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Introduction

Toll-like receptors (TLRs) recognize molecules derived from pathogens and play crucial roles in the innate immune system. TLRs are type I integral membrane glycoproteins characterized by the extracellular domains with varying numbers of leucine-rich-repeat motifs (LRR) and a cytoplasmic signaling domain termed the Toll/interleukin 1 receptor (TIR) domain (Akira et al. 2006; Bowie and O'Neill 2000). Different TLRs recognize a variety of

pathogen-associated molecules, including lipids and nucleic acids, and all TLRs transduce signals through TIR domains to activate immune cells (Akira et al. 2006; Bowie and O'Neill 2000). Stimulation with TLR ligands recruits adaptor proteins such as Myeloid differentiation factor 88 (MYD88), TIR domain-containing adaptor protein (TIRAP), TIR domain-containing adaptor molecule 1 (TICAM1), and TICAM2, all of which also have a TIR domain, to the cytoplasmic portion of the TLRs and activate signaling cascades to produce proinflammatory cytokines and chemokines (Akira et al. 2006; Bowie and O'Neill 2000).

Viral, bacterial, and parasitic infections have been postulated to be among the strongest selective pressures on primate evolution. It is also widely accepted that the susceptibility to infectious pathogens, such as *Mycobacterium tuberculosis* bacilli and HIV-1, are different among primate species (Isaza 2003; Stremlau et al. 2004). Given that TLRs play crucial roles in the innate immune system, the intriguing hypothesis that TLRs have emerged under the intense pressure of natural selection in the course of primate evolution is rising. Actually, it is suggested that *TLR1*, *TLR6*, and *TLR10* have come under particular natural selection pressures in the human population, because the sequence variations of these three genes display considerable geographical diversity in the British population (Wellcome Trust Case Control Consortium 2007). Moreover, it has been reported that natural selection has acted on *TLR4* in humans, since excess of rare non-synonymous polymorphisms in *TLR4* are observed in humans (Smirnova et al. 2001).

To investigate the natural selection hypothesis, we analyzed the nucleotide sequences of 16 TLR-related genes, including ten *TLRs* (TLR1–10), four genes linked to signal transduction (*MYD88*, *TILAP*, *TICAM1*, and *TICAM2*), and two genes linked to *TLR4* (*MD2* and *CD14*) in primates. MD2 and CD14 are key molecules of the LPS signaling through TLR4 (Poltorak et al. 1998; Shimazu et al. 1999; Nagai et al. 2002). Our study shows that the genomic segments encoding the intracellular TIR domains have undergone purifying selection and that the extracellular domain of *TLR4* has been the suggestive target of positive Darwinian selection in the course of primate evolution. We concluded that natural selection has indeed shaped the sequence patterns of TLR-related genes in primate evolution.

Materials and methods

DNA sequences

DNA samples from 25 primates, including human (*Homo sapiens*), chimpanzee (*Pan troglodytes*), bonobo (*Pan paniscus*), gorilla (*Gorilla gorilla*), orangutan (*Pongo pygmaeus*), black gibbon (*Hylobates concolor*), white-handed gibbon (*Hylobates lar*), siamang (*Hylobates syndactylus*), crab-eating macaque (*Macaca fascicularis*), rhesus macaque (*Macaca mulatta*), hamadryas baboon (*Papio hamadryas*), black and white colobus (*Colobus guereza*), silvered lutong (*Trachypithecus cristatus*), dusky lutong (*Trachypithecus obscurus*), common marmoset (*Callithrix jacchus*), cotton-top tamarin (*Saguinus oedipus*), red-handed tamarin (*Saguinus midas*), lion tamarin (*Leontopithecus rosalia*), common squirrel monkey (*Saimiri sciureus*), tufted capuchin (*Cebus apella*), long-haired spider monkey (*Ateles belzebuth*), and Central American spider monkey (*Ateles geoffroyi*), tarsiers (*Tarsius* spp.), lesser galago (*Galago senegalensis*), and ring-tailed lemur (*Lemur catta*) were analyzed. Overlapping primer sets covering all coding exons of 16 genes including the *TLRs* (*TLR1–10*), *MYD88*, *TILAP*, *TICAM1*, *TICAM2*, *MD2*, and *CD14* were designed on the basis of size and overlap of PCR amplicons. Genomic DNA was subjected to PCR amplification followed by sequencing using the BigDye Terminator cycling system. Sequencing analysis was performed in an ABI3130x automated DNA sequencer (Applied Biosystems).

Statistical analysis

Sequence alignments were performed by the Clustal X program (Thompson et al. 1997). All values for Ka, Ks, Ka/Ks, %GC, and Codon Bias Index (CBI) were evaluated by DnaSP (Rozas et al. 2003). The Bn–Bs program (Zhang et al. 1998) was applied to evaluate the Ka/Ks ratio in individual branches of the primate phylogenetic tree. We studied positive Darwinian selection for the target region of TLR4 by using the MEGA version 4.0 program (Tamura et al. 2007). Ancestral amino acid sequence was estimated by a parsimony method using PROTPARS program in PHYLIP (Felsenstein 1989).

Results

The nucleotide sequences of tn *TLRs* (TLR1–10), *MYD88*, *TILAP*, *TICAM1*, *TICAM2*, *MD2*, and *CD14* were determined among seven primates, including human, chimpanzee, bonobo, gorilla, orangutan, crab-eating macaque, and rhesus macaque. All sequences were newly determined in the study, and all accession numbers were shown in Table S1. The lengths of the deduced coding sequences for each gene differed among the seven primates, as summarized in Table 1.

To evaluate the non-synonymous/synonymous substitution ratio, we applied the Bn–Bs program (Zhang et al. 1998). This program uses a modified Nei–Gojobori method (Nei and Gojobori 1986) to estimate pairwise synonymous