Supplemental Figure S1

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human:                          ATGCATCCTC AAGTGGTCAT CTTAAGCCTC ATCCTAC--- ---------- ---------- ---------- ---------- ----------
common marmoset:                CAGCATCCTC AAGTGGTCAT CTTAAGCCTC ATCCTAC--- ---TTTTTTT TTAATTGCAT TTTAGGTTTT GGGGTACATG TGAAGAACAT
golden lion tamarin:            ATGCATCCTT AAGTGGTCAT CTTAAGCCTC ATTCTACTTC TTTTTTTTTT TTTATTGCCT TTTAGGTTTT GGGGTACATG TGAAGAACAT
cotton-top tamarin:             ATGCATCCTC AAGTGGTTGT CTTAAGCCTT ATCCTAC--- -----TTTTT TTAATTGCCT TTTAGGCTTT GGGGTACATG TGAAGAACAT
white-lipped tamarin:           ATGCATCCTC AAGTGGTTGT CTTAAGCCTT ATCCTAC--- ----TTTTTT TTTATTGCCT TTTAGGCTTT GGGGTACATG TGAAGAACAT
golden-handed tamarin:          ATGCATCCTC AAGTGGTTGT CTTAAGCCTT ATCCTAC--- -TTTTTTTTT TTTATTGCCT TTTAGGCTTT GGGGTACATG TGAAGAACAT
common squirrel monkey:         ATGCCTCCTC AAGGGGTCGT CTTAAGCCTC ATCCTAC--- ---------- ---------- ---------- ---------- ----------
tufted capuchin:                ATGCATCCTC AGGTGGTCGT CTTAAGCCTC ATGCTAC--- ---------- ---------- ---------- ---------- ----------
white-fronted spider monkey:    ATGCATCCTC AAGTGGTCGT CTTAAGCCTC ATCCTAC--- ---------- ---------- ---------- ---------- ----------
Geoffroy's spider monkey:       ATGCATCCTC AAGTGGTTGT CTTAAGCCTC ATCCTAC--- ---------- ---------- ---------- ---------- ----------

human:                          ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
common marmoset:                GCAAGATAGT TGCATAGGTA CACATGTGGC AGTGTGATTT GCTGCCTTCC TCCCCTTCAC CTATA-CTGG CATTTTCCCC CATGCTATCT
golden lion tamarin:            GCAAGATAGT TGCATAGGTA CACACGCGGC AGTGTGATTT GCTGC-TTCC TCCCCTTCAC CTATATCTGG CATTTTTCCC CATGCTCTCT
cotton-top tamarin:             GCAAGATAGT TGCATAGATA CACACGTGGC AATGTGATTT GCTGCCTTCC TCCCCTTCAC CTATATCTGG CATTTTTCCC CATGCTCTCT
white-lipped tamarin:           GCAAGATAGT TGCATAGATA CACACGTGGC AGTGTGATTT GCTGCCTTCC TCCCCTTCAC CTATATCTGG CATTTTTCCC CATGCTCTCT
golden-handed tamarin:          GCAAGATAGT TGCATAGATA CACACGTGGC AATGTGATTT GCTGCCTTCC TCCCCTTCAC CTATATCTGG CATTTTTCCC CATGCTCTCT
common squirrel monkey:         ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
tufted capuchin:                ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
white-fronted spider monkey:    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Geoffroy's spider monkey:       ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

human:                          ---------- ---------- ---------- ---------- ---------- ---------- ---------- -ATCTGGCAG
common marmoset:                CT-CCCCAAC TACCCACCCC CGCTGTCCC- TCCCCATCAT TTTCAGCAAA CTGACACAAG CCTCATCCTA CTTCTAGCAG
golden lion tamarin:            CTCCCCAACT CCCCGCCCCC CGCTGTCCC- TCCCCATCAT TCTCAGCAAA CTGACACAAG CCTCATCCTA CTTCTAGCAG
cotton-top tamarin:             TTCCCCAACT CCCAGCCCCC CGCTGTCCCC TCCCCATCAT TCTCAGCAAA CTGACACGAG CCTCATCCTA CTTCTAGCAA
white-lipped tamarin:           CTCCCCAACT CCCAACCCCC CGCTGTCCCC TCCCCATCAT TCTCAGCAAA CTGACAGGAG CCTTATCCTA CTTCTAGCAG
red-handed tamarin:             CTCCCCAACT CCCAACCCCC CGCTGTCCCC TCCCCATCAT TCTCAGCAAA CTGACAGGAG CCTTATCCTA CTTCTAGCAG
common squirrel monkey:         ---------- ---------- ---------- ---------- ---------- ---------- ---------- -TTCTAGCAG
tufted capuchin:                ---------- ---------- ---------- ---------- ---------- ---------- ---------- -TTCTAGCAG
white-fronted spider monkey:    ---------- ---------- ---------- ---------- ---------- ---------- ---------- -TTCTAGCAG
Geoffroy's spider monkey:       ---------- ---------- ---------- ---------- ---------- ---------- ---------- -TTCTAGCAG
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Supplemental Figure S2

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Human TIM1:          MHPQVVILSL ILHLADSVAG SVKVGGEAGP SVTLPCHYSG AVTSMCWNRG SCSLFTCQNG IVWTNGTHVT YRKDTRYKL-
Marmoset TIM1-like: MPPQVVILSL ILLLAD-ALV SLQVGGVAGP STMLPCSYSG DVTSMC*NRD RCSLLRCPNS IIWTNGTHVT YHCAVNYML*

Human TIM1:          -LGDLSRRDV SLTIENTAVS DSGVYCCRVE HRGWFNDMKI TVSLEI---- -----VP--- ---------- ----------
Marmoset TIM1-like: TMGDLSKRDV SLTLGALWEA EVGGSQGQEI ETSLGNIVKT LSLLKI*KLS QAWWHVPVVQ LLGRLRQENC LNPRGRACSK

Human TIM1:          ---PKVTTTP IVTTVPTVTT VRTSTTVPTT TTVPMTTVPT TTVPTTMSIP TTTTVLTTMT VSTT---TSV PTTTSIPTTT
Marmoset TIM1-like: PRSHHCTPTW QQSETPSPTK KRKENTSLSD SGLYCCHVGH KV*FNDMKI- TVSLAMVPPR VTTTPIVTIV PTFTTVRMST

Human TIM1:          SVPVTTTVST FVPPMPLPRQ NHEPVATSPS SPQPAETHPT TLQGAIRREP TSSPLYSYTT DGNDTVTESS DGLWNNNQTQ
Marmoset TIM1-like: TVPTTMTVSS FAPPTPSPTQ NHGP-ATPPS SPQPTETHPA MLQEATRTQR AGSPLHSYTT NGNDTVTESS DGLWNNDQTQ

Human TIM1:          LFLEHSLLTA NTTKGIYAGV CISVLVLLAL LGVIIAKKYF FKKEVQQLSV SFSSLQIKAL QNAVEKEVQA EDNIYIENSL YATD-
Marmoset TIM1-like: LSPAQSPQMA TPTKGICAGV CMPVLVPLAL LGVIIARKYF FRNKI*QLSF SFRRLQIKAL QNAVKKEVQA EDSVYVENNL YATDS
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Association of MHC-I genotypes with disease progression in HIV/SIV infections

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Virus-specific cytotoxic T lymphocytes (CTLs) are major effectors in acquired immune responses against viral infection. Virus-specific CTLs recognize specific viral peptides presented by major histocompatibility complex class-I (MHC-I) on the surface of virus-infected target cells via their T cell receptor (TCR) and eliminate target cells by both direct and indirect mechanisms. In human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) infections, host immune responses fail to contain the virus and allow persistent viral replication, leading to AIDS progression. CTL responses exert strong suppressive pressure on HIV/SIV replication and cumulative studies have indicated association of HLA/MHC-I genotypes with rapid or slow AIDS progression.

Keywords: CTL, HIV, HLA, Mamu, MHC-I, MHC-I haplotype, SIV

INTRODUCTION

Innate and acquired immune responses play an important role in the control of infectious pathogens. Pathogenic microbes are able to escape from the host innate immune responses and replicate in the hosts. After the acute growth phase, pathogen-specific neutralizing antibody and cytotoxic T lymphocyte (CTL) responses are induced and prevent the onset of pathogenic manifestations in most of acute infectious diseases. In HIV and simian immunodeficiency virus (SIV) infections, these acquired immune responses are induced but fail to contain the virus and allow persistent viral replication, leading to AIDS progression, while persistent SIVsm infection of natural hosts, sooty mangabeys, does not result in disease onset (Silvestri et al., 2003). Effective neutralizing antibody responses are not efficiently induced in the acute phase (Burton et al., 2004). In contrast, virus-specific CTL responses play a main role in the reduction of viral loads from the peak to the set-point levels (Borrow et al., 1994; Koup et al., 1994; Matano et al., 1998; Jin et al., 1999; Schmitz et al., 1999). Previous studies suggest that, among various viral antigen-specific CTL responses, those directed against the viral structural protein Gag contribute to the control of viral replication (Edwards et al., 2002; Zuniga et al., 2006; Borghans et al., 2007; Kiepiela et al., 2007).

In virus-infected cells, antigenic peptides that are processed from viral proteins via the proteasome pathway and bound to MHC-I (HLA class I) molecules are presented on the cell surface. CTLs recognize antigenic peptide (epitope)-MHC-I complexes on the cell surface by their TCRs and eliminate the virus-infected cells by inducing apoptosis or lysis. Because presentation of antigenic peptides is restricted by MHC-I molecules, CTL efficacy is affected by MHC-I (HLA class I) genotypes.

ASSOCIATION OF HLA ALLELES WITH HIV PROGRESSION

HIV-infected individuals without anti-retroviral therapy (ART) mostly develop AIDS in 5–10 years after HIV exposure (Lui et al., 1988; Farewell et al., 1992). Humans have a single polymorphic HLA-A, HLA-B, and HLA-C locus per chromosome. A number of studies on HIV-infected individuals reported the association of HLA genotypes with disease progression (Tang et al., 2002; Kiepiela et al., 2004; Wang et al., 2009; Leslie et al., 2010). Indeed, association of *HLA-B*57* (Migueles et al., 2000; Altfeld et al., 2003; Miura et al., 2009) and *HLA-B*27* (Goulder et al., 1997; Feeney et al., 2004; Altfeld et al., 2006; Schneidewind et al., 2007) with lower viral loads in the chronic phase and slow disease progression has been indicated. HLA-B*57-restricted $Gag_{240-249}$ TW10 (TSTLQEQIGW) and HLA-B*27-restricted $Gag_{263-272}$ KK10 (KRWIILGLNK) epitope-specific CTL responses exert strong suppressive pressure on HIV replication and often select for viral genome mutations resulting in viral escape from these CTL recognition with viral fitness costs (Goulder et al., 1997; Feeney et al., 2004). Some HIV-infected individuals possessing those HLA alleles associating with slower disease progression control viral replication for long periods, while the frequency of such elite controllers is under 1% (Lambotte et al., 2005; Grabar et al., 2009). In contrast, HLA genotypes such as *HLA-B*35* associating with rapid disease progression have also been reported (Carrington et al., 1999; Gao et al., 2001). *HLA-B*35* subtypes are divided into *HLA-B*35-Px* and *HLA-B*35-Py* based on the specificity of binding ability to epitope peptides in the P9 pocket. The former group, *HLA-B*35-Px* alleles including *HLA-B*3502*, *B*3503*, and *B*3504* associate with rapid disease progression, whereas the latter *HLA-B*35-Py* alleles including *HLA-B*3501* and *HLA-B*3508* associate with relatively slower progression (Gao et al., 2001). Such differences in disease progression among *HLA-B* subtypes are also known in *HLA-B*58* (Leslie et al., 2010).

ANIMAL AIDS MODELS

Robust non-human primate AIDS models showing high pathogenic homology to human HIV infections are essential for

AIDS research. While it is difficult to analyze the early phase in human HIV infection, animal models have considerable advantages in immunological analysis in the acute phase. Furthermore, comparisons among the hosts infected with the same virus strain are possible in animal AIDS models, although highly diversified HIVs are prevalent in humans. An important characteristic of HIV infection is selective loss of memory $CCR5^+$ $CD4^+$ T lymphocytes in the acute phase leading to persistent virus replication (Connor et al., 1997; Zhang et al., 1999; Brenchley et al., 2004). HIV tropism for $CCR5^+$ $CD4^+$ memory cells is considered as one central mechanism for persistent infection. R5-tropic SIVmac251/SIVmac239 or SIVsmE660/SIVsmE543-3 infection of rhesus macaques inducing the acute, selective loss of memory $CD4^+$ T lymphocytes is currently considered the best AIDS model for analysis of AIDS pathogenesis and evaluation of vaccine efficacy (Veazey et al., 1998; Nishimura et al., 2004; Bontrop and Watkins, 2005; Mattapallil et al., 2005; Morgan et al., 2008). Recent studies indicated an association of restriction factor TRIM5α genotypes with disease progression in macaques infected with pathogenic SIVs such as SIVsmE660/SIVsmE543-3 but not in SIVmac239 infection (Kirmaier et al., 2010; Lim et al., 2010; de Groot et al., 2011; Fenizia et al., 2011; Letvin et al., 2011; Reynolds et al., 2011; Yeh et al., 2011). Macaque AIDS models of chimeric simian-human immunodeficiency virus (SHIV) infection are also known. Infection with X4-tropic SHIVs such as SHIV89.6P results in acute $CD4^+$ T cell depletion, while R5-tropic SHIVs such as SHIV162P3 induce persistent infection leading to chronic disease progression (Tsai et al., 2007; Nishimura et al., 2010; Zhuang et al., 2011). These SHIVs are useful especially for the analysis of Env-specific antibody responses (Ng et al., 2010; Watkins et al., 2011).

GENETIC FEATURES OF MHC-I IN MACAQUES

Human classical MHC-I alleles are composed of a single polymorphic HLA-A, HLA-B, and HLA-C locus per chromosome. MHC-I haplotypes in rhesus macaques, however, have variable numbers of Mamu-A and Mamu-B loci (Boyson et al., 1996; Adams and Parham, 2001; Daza-Vamenta et al., 2004; Kulski et al., 2004; Otting et al., 2005; **Figure 1**). A number of studies described SIV infections in macaques sharing one or two MHC-I alleles, while few studies have examined SIV infection in macaques sharing an MHC-I haplotype.

PROTECTIVE MHC-I ALLELES IN INDIAN RHESUS MACAQUES AGAINST SIV INFECTION

Simian immunodeficiency virus infections of Indian rhesus macaques are widely used as an AIDS model. *Mamu-A*01*, *Mamu-B*08*, and *Mamu-B*17* are known as protective alleles and macaques possessing these alleles tend to show slow disease progression after SIVmac251/SIVmac239 challenge (Muhl et al., 2002; Mothe et al., 2003; Yant et al., 2006; Loffredo et al., 2007b). Fourteen Mamu-A*01-restricted SIVmac239 CTL epitopes have been reported (Allen et al., 2001; Mothe et al., 2002b). Mamu-A*01-restricted Tat_{28-35} SL8 (STPESANL)-specific and $Gag_{181-189}$ CM9 (CTPYDINQM)-specific CTL responses are induced dominantly in SIVmac239 infection. Both epitope-specific CTLs show strong suppressive capacity against SIVmac239 replication

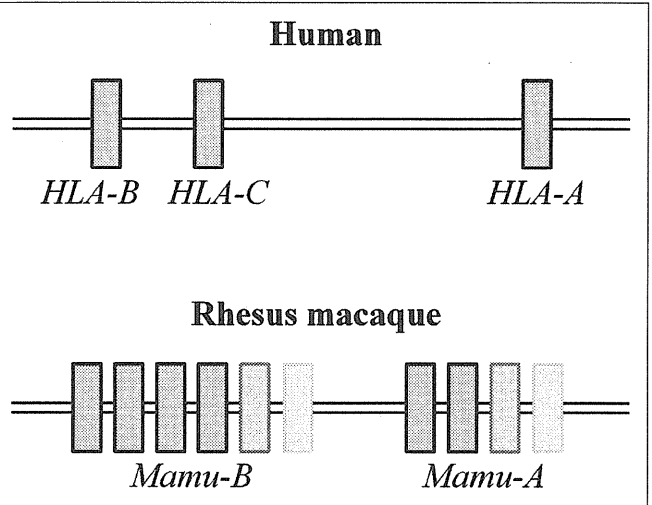

FIGURE 1 | Comparison of genome structures of MHC-I sub-regions in humans and rhesus macaques. Humans have a single polymorphic HLA-A, HLA-B, and HLA-C locus per chromosome, while rhesus macaques have variable numbers of Mamu-A and Mamu-B loci per chromosome.

in vitro (Loffredo et al., 2005), while the latter but not the former play a major role in suppression of viral replication *in vivo* (O'Connor et al., 2002; Loffredo et al., 2007c). In SHIV89.6P infection, Mamu-A*01-positive macaques elicit CM9-specific CTL responses and show slower disease progression than Mamu-A*01-negative animals (Zhang et al., 2002). Eight Mamu-B*08-restricted SIVmac239 CTL epitopes have been reported; previous studies indicated that $Vif_{123-131}$ RL9 (RRAIRGEQL), $Vif_{172-179}$ RL8 (RRDNRRGL), and $Nef_{137-146}$ RL10 (RRHRILDIYL) epitope-specific CTL responses contribute to viral control (Loffredo et al., 2007a; Loffredo et al., 2008; Valentine et al., 2009; Mudd et al., 2012). SIVmac239 Vif_{66-73} HW8 (HLEVQGYW), $Nef_{165-173}$ IW9 (IRYPKTFGW), and $Nef_{195-203}$ MW9 (MHPAQTSQW) have been reported as Mamu-B*17-restricted CTL epitopes (Mothe et al., 2002a). In addition, cRW9 (RHLAFKCLW) in an alternate reading frame is known as a cryptic epitope (Maness et al., 2007). The cRW9-coding region [nucleotides 6889–6915 in SIVmac239 (accession number M33262)] is located in the same open reading frame that encodes exon 1 of the Rev protein but is downstream of the splice donor site. So, it is not predicted to be translated under normal biological circumstances. However, SIVmac239-infected Mamu-B*17-positive macaques efficiently induce cRW9-specific CTL responses.

ASSOCIATION OF MHC-I HAPLOTYPES WITH DISEASE PROGRESSION AFTER SIVmac239 CHALLENGE IN BURMESE RHESUS MACAQUES

We accumulated groups of Burmese rhesus macaques sharing individual MHC-I haplotypes (Tanaka-Takahashi et al., 2007; Naruse et al., 2010). SIVmac239 challenge of Burmese rhesus macaques mostly results in persistent viremia (geometric means of setpoint plasma viral loads: about 10^5 copies/ml) leading to AIDS (mean survival periods: about 2 years; Nomura et al., 2012). Further analysis revealed the association of MHC-I haplotypes with disease progression after SIVmac239 challenge.

Table 1 | Association of MHC-I haplotypes with disease progression in SIV infection (Nomura et al., 2012).

MHC-I haplotypes	Mean survival periods	Geometric means of setpoint plasma viral loads (copies/ml)	Peripheral CD4$^+$ T cell decline	Predominant CTL responses
90-120-Ia	>40 months	10^4	Slow	Gag/Nef
90-010-Ie	23 months	10^5	Intermediate	Nef
90-120-Ib	24 months	10^5	Intermediate	Nef
90-088-Ij	15 months	10^6	Rapid	-

In our study (Nomura et al., 2012), the group of Burmese rhesus macaques possessing MHC-I haplotype *90-010-Ie* (dominant MHC-I alleles: *A1*066:01* and *B*005:02*) exhibited a typical pattern of disease progression after SIVmac239 challenge (**Table 1**). These animals showed predominant Nef-specific CTL responses, approximately 10^5 copies/ml of setpoint plasma viral loads (geometric means), and 2 years of mean survival periods. Another group of macaques possessing *90-120-Ib* (dominant MHC-I alleles: *A1*018:08* and *B*036:03*) showed similar setpoint viral loads and survival periods. However, the group of Burmese rhesus macaques possessing MHC-I haplotype *90-088-Ij* (dominant MHC-I alleles: *A1*008:01* and *B*007:02*) showed higher setpoint plasma viral loads (geometric means: about 10^6 copies/ml) and shorter survival periods (means: about 15 months; **Table 1**). These animals mostly showed poor CTL responses.

In contrast, the group of Burmese rhesus macaques possessing MHC-I haplotype *90-120-Ia* (dominant MHC-I alleles: *A1*043:01* and *B*061:03*), referred to as A$^+$ animals, showed lower setpoint plasma viral loads (geometric means: about 10^4 copies/ml) and slower disease progression (means of survival periods: more than 40 months; **Table 1**). These animals predominantly elicited Gag-specific and Nef-specific CTL responses after SIVmac239 challenge. Mamu-A1*043:01-restricted $Gag_{206-216}$ (IINEEAADWDL) and Mamu-A1*065:01-restricted $Gag_{241-249}$ (SSVDEQIQW) were determined as dominant CTL epitopes. SIVmac239-infected A$^+$ animals selected viral escape mutations from these epitope-specific CTL responses with viral fitness costs in the chronic phase (Kobayashi et al., 2005; Kawada et al., 2006). These mutations are GagL216S, a mutation leading to a leucine (L)-to-serine (S) substitution at the 216th amino acid in SIVmac239 Gag, and GagD244E, aspartic acid (D)-to-glutamic acid (E) at the 244th, or GagI247L, isoleucine [I]-to-L at the 247th. A$^+$ animals immunized with a prophylactic prime-boost vaccine consisting of a DNA prime followed by a boost with a recombinant Sendai virus vector expressing SIVmac239 Gag controlled an SIVmac239 challenge (Matano et al., 2004). However, vaccinated A$^+$ animals failed to control a challenge with a mutant SIVmac239 carrying GagL216S and GagD244E, indicating that $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses are responsible for the control of the wild-type SIVmac239 replication (Kawada et al., 2006, 2008). Interestingly, the Mamu-A1*065:01-restricted SIVmac239 $Gag_{241-249}$ epitope is located in a region corresponding to the HLA-B*57-restricted HIV $Gag_{240-249}$ epitope TW10 and TW10-specific CTL responses have also been indicated to exert strong suppressive pressure on HIV replication. An SIVmac239 $Gag_{241-249}$-specific CTL escape mutation, GagD244E, results in loss of viral fitness similarly with an HIV TW10-specific CTL escape mutation. Both of the Mamu-A1*065:01-restricted SIVmac239 $Gag_{241-249}$ epitope and the HLA-B*57-restricted HIV TW10 epitope are considered to have the same anchor residues, S at position 2 and tryptophan (W) at the carboxyl terminus. Additionally, anchor residues of CTL epitopes presented by Mamu-B*17/Mamu-B*08 were indicated to be similar to those restricted by HLA-B*57/HLA-B*27 (Loffredo et al., 2009; Wu et al., 2011).

CONCLUDING REMARKS

Human HLA genotypes largely affect disease progression in HIV infection, reflecting that CTL responses play a central role in suppression of HIV replication. Animal AIDS models are required for understanding of the interaction between highly diversified viruses and the hosts with polymorphic MHC-I genotypes. SIV infection of Indian rhesus macaques are widely used as an AIDS model, and association of certain MHC-I alleles with slower disease progression has been indicated. We have recently reported SIV infection of Burmese rhesus macaques as a robust AIDS model and indicated association of MHC-I haplotypes with disease progression. Accumulation of those macaque groups sharing MHC-I haplotypes could lead to constitution of a more sophisticated AIDS model facilitating analysis of virus-host immune interaction.

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Original article

Immunogenicity of repeated Sendai viral vector vaccination in macaques

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Abstract

Induction of durable cellular immune responses by vaccination is an important strategy for the control of persistent pathogen infection. Viral vectors are promising vaccine tools for eliciting antigen-specific T-cell responses. Repeated vaccination may contribute to durable memory T-cell induction, but anti-vector antibodies could be an obstacle to its efficacy. We previously developed a Sendai virus (SeV) vector vaccine and showed the potential of this vector for efficient T-cell induction in macaques. Here, we examined whether repeated SeV vector vaccination with short intervals can enhance antigen-specific $CD8^+$ T-cell responses. Four rhesus macaques possessing the MHC-I haplotype 90-120-Ia were immunized three times with intervals of three weeks. For the vaccination, we used replication-defective F-deleted SeV vectors inducing $CD8^+$ T-cell responses specific for simian immunodeficiency virus $Gag_{206-216}$ and $Gag_{241-249}$, which are dominant epitopes restricted by 90-120-Ia-derived MHC-I molecules. All four animals showed higher $Gag_{206-216}$-specific and $Gag_{241-249}$-specific $CD8^+$ T-cell responses after the third vaccination than those after the first vaccination, indicating enhancement of antigen-specific $CD8^+$ T-cell responses by the second/third SeV vector vaccination even with short intervals. These results suggest that repeated SeV vector vaccination can contribute to induction of efficient and durable T-cell responses.

Keywords: AIDS vaccine; Viral vector; CTL; SIV

1. Introduction

Antigen-specific T-cell responses play a central role in the control of persistent infection with pathogens such as human immunodeficiency viruses (HIVs) [1–7]. Induction of efficient and durable T-cell responses is an important vaccine strategy, and recombinant viral vectors are promising vaccine tools for antigen-specific T-cell induction [8]. Many kinds of viral vectors including adenovirus (AdV) and poxvirus vectors have been shown to efficiently induce antigen-specific T-cell responses [9–14]. Repeated viral vector vaccination may induce enhanced and durable memory T-cell responses. Viral vector vaccination, however, elicits antibodies against the vector virus itself, which could be an obstacle to the potential of repeated viral vector vaccination [15].

We previously developed a vaccine system using Sendai virus (SeV) vectors to induce antigen-specific T-cell responses [16,17]. We have replication-defective (nontransmissible) F-deleted SeV, F(-)SeV, as well as replication-competent SeV vectors [18,19]. In our recent study [20], intranasal immunization even with a lower dose (6×10^8 CIU [cell infectious units]) of F(-)SeV vectors in SeV-infected macaques efficiently elicited antigen-specific $CD8^+$ T-cell responses in the presence of SeV-specific neutralizing titers of 1:50–1:100.

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Intramuscular F(-)SeV vector immunization can also induce antigen-specific $CD8^+$ T-cell responses efficiently in the absence of anti-SeV neutralizing antibodies, but intranasal F(-)SeV vector vaccination is more immunogenic than intramuscular in the presence of anti-SeV neutralizing antibodies.

Thus, repeated intranasal SeV vector immunization may have the potential to overcome anti-SeV antibody responses and induce more efficient and durable T-cell responses than those by single immunization. Our previous analyses in macaques [21] showed efficient antigen-specific $CD8^+$ T-cell induction by an intranasal immunization with 6×10^9 CIU of F(-)SeV vectors more than one year after an initial SeV vector inoculation, indicating the immunogenicity of repeated SeV vector vaccination with long intervals.

In the present study, we investigated whether repeated SeV vector vaccination with short intervals can enhance antigen-specific $CD8^+$ T-cell responses. Macaques received SeV vectors intranasally and intramuscularly at the second and the third vaccination at weeks 3 and 6 after the first intranasal SeV vector vaccination. While clear difference in immunogenicity was not shown between intranasal and intramuscular SeV vector administration, all the animals exhibited higher antigen-specific $CD8^+$ T-cell responses after the third vaccination than those after the first. Our results indicate that repeated SeV vector vaccination even with short intervals can contribute to induction of efficient, durable T-cell responses.

2. Materials and methods

2.1. Animals

The animal experiments were conducted through the Cooperative Research Program in Tsukuba Primate Research Center (TPRC), National Institute of Biomedical Innovation with the help of the Corporation for Production and Research of Laboratory Primates. All animals were maintained in accordance with the guidelines for laboratory animals of the National Institute of Infectious Diseases and TPRC. Blood collection and vaccination were performed under ketamine anesthesia.

In the present study, we used four Burmese rhesus macaques possessing the major histocompatibility complex class I (MHC-I) haplotype *90-120-Ia* [22]; those *90-120-Ia*-positive macaques are known to dominantly elicit SIVmac239 $Gag_{206-216}$ (IINEEAADWDL) epitope-specific and $Gag_{241-249}$ (SSVDEQIQW) epitope-specific $CD8^+$ T-cell responses after SIVmac239 challenge [23,24]. In our previous study [25–27], these four macaques were challenged with SIV after vaccination and controlled SIV replication in the chronic phase as follows. Macaque R04-016 received a prophylactic prime-boost vaccine eliciting single $Gag_{241-249}$ epitope-specific $CD8^+$ T-cell responses before SIVmac239 challenge [26]. Macaque R04-015 received a prophylactic prime-boost vaccine eliciting $Gag_{206-216}$ epitope-specific and $Gag_{241-249}$ epitope-specific $CD8^+$ T-cell responses [27,28]. Macaques R06-015 and R06-035 received a prophylactic DNA prime/F(-)SeV-Gag boost vaccine [25].

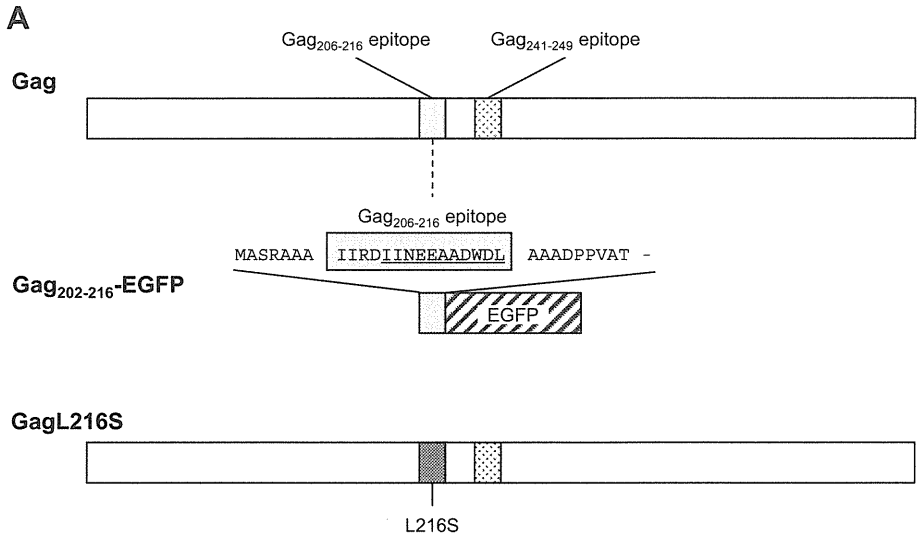
Macaques R04-015, R04-016, and R06-015 contained a challenge with SIVmac239 [29] approximately 3 months after the boost and a superchallenge with a mutant SIVmac239 carrying five gag mutations, SIVmac239Gag216S244E247L312V373T, in the chronic phase (at week 40 [R06-015] or 116 [R04-015 and R04-016] after SIVmac239 challenge) [27]. Macaque R06-035 was challenged with a mutant SIVmac239, SIVmac239-Gag216S244E, carrying two gag mutations, GagL216S and GagD244E leading to a leucine (L)-to-serine (S) substitution at the 216th amino acid and an aspartic acid (D)-to-glutamic acid (E) substitution at the 244th in Gag [25]. These mutations result in escape from $Gag_{206-216}$-specific and $Gag_{241-249}$-specific $CD8^+$ T-cell responses. In this animal that failed to control the mutant SIV challenge, persistent viremia was observed for 8 months, but after that, plasma viremia became undetectable.

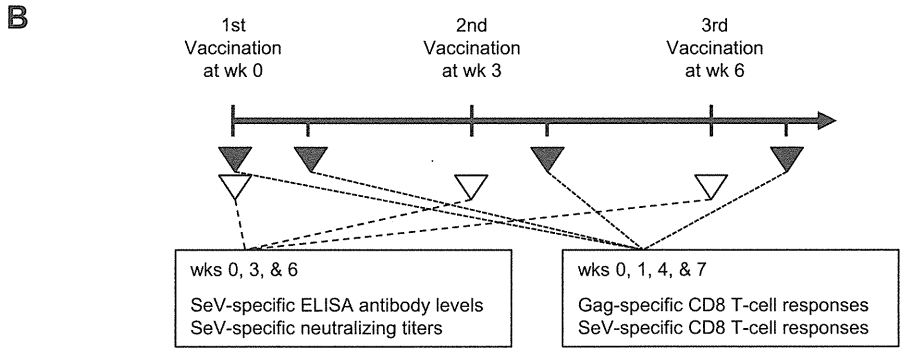
2.2. Vaccination

In the present study, macaques R04-015, R04-016, R06-015, and R06-035 received an intranasal F(-)SeV-Gag vector vaccination (referred to as the first vaccination) at weeks 128, 128, 52, and 67 after SIV challenge, respectively. At the second and the third vaccination, we used two kinds of F(-)SeV vectors, F(-)SeV-$Gag_{202-216}$-EGFP expressing $Gag_{202-216}$-EGFP fusion protein and F(-)SeV-Gag216S expressing SIVmac239 Gag with a mutation leading to escape from $Gag_{206-216}$-specific $CD8^+$ T-cell responses [23], for eliciting $Gag_{206-216}$-specific and $Gag_{241-249}$-specific $CD8^+$ T-cell responses, respectively (Fig. 1). The F(-)SeV-Gag216S is considered not to induce $Gag_{206-216}$-specific $CD8^+$ T-cell responses, because challenge of macaques with a SIV carrying this Gag216S mutation induced no $Gag_{206-216}$-specific $CD8^+$ T-cell responses in a previous study [25]. Macaques R04-015 and R06-035 received F(-)SeV-$Gag_{202-216}$-EGFP intranasally and F(-)SeV-Gag216S intramuscularly at the second vaccination three weeks after the first. At the third vaccination three weeks after the second, these animals received F(-)SeV-Gag216S intranasally and F(-)SeV-$Gag_{202-216}$-EGFP intramuscularly. On the contrary, macaques R04-016 and R06-015 received F(-)SeV-Gag216S intranasally and F(-)SeV-$Gag_{202-216}$-EGFP intramuscularly at the second vaccination and F(-)SeV-$Gag_{202-216}$-EGFP intranasally and F(-)SeV-Gag216S intramuscularly at the third. The dose of each vaccination was 6×10^9 cell infectious units (CIU). During the observation period in the present study, plasma viremia was undetectable in all four macaques.

2.3. Measurement of antigen-specific $CD8^+$ T-cell responses

We measured antigen-specific $CD8^+$ T-cell frequencies by flow-cytometric analysis of gamma interferon (IFN-γ) induction after specific stimulation as described previously [21,27]. Peripheral blood mononuclear cells (PBMCs) were cocultured with autologous herpesvirus papio-immortalized B lymphoblastoid cell lines (B-LCLs) pulsed with 1 μM $Gag_{206-216}$ or $Gag_{241-249}$ peptide for $Gag_{206-216}$-specific or $Gag_{241-249}$-specific stimulation. For SeV-specific stimulation, PBMCs were cocultured with B-LCLs infected with SeV. Intracellular IFN-γ staining was performed using CytofixCytoperm kit





animal ID	1st Vaccination at wk 0	2nd Vaccination at wk 3	3rd Vaccination at wk 6
R04-015 R06-035	F(-)SeV-Gag IN	F(-)SeV-Gag$_{202-216}$-EGFP IN F(-)SeV-Gag216S IM	F(-)SeV-Gag216S IN F(-)SeV-Gag$_{202-216}$-EGFP IM
R04-016 R06-015	F(-)SeV-Gag IN	F(-)SeV-Gag216S IN F(-)SeV-Gag$_{202-216}$-EGFP IM	F(-)SeV-Gag$_{202-216}$-EGFP IN F(-)SeV-Gag216S IM

Fig. 1. Vaccination protocols. (A) Inserts of F(-)SeV vectors used for vaccination. F(-)SeV-Gag, F(-)SeV-Gag$_{202-216}$-EGFP, and F(-)SeV-Gag216S express the SIVmac239 Gag, a Gag$_{202-216}$-EGFP fusion protein, and a mutant Gag with a leucine-to-serine substitution at the 216th amino acid (L216S), respectively. The L216S substitution results in escape from Gag$_{206-216}$-specific CD8$^+$ T-cell recognition. (B) Protocols for macaque vaccination. Four *90-120-Ia*-positive macaques received F(-)SeV-Gag intranasally (IN) at the first vaccination. Macaques R04-015 and R06-035 received F(-)SeV-Gag$_{202-216}$-EGFP IN and F(-)SeV-Gag216S intramuscularly (IM) at the second vaccination and vice versa at the third. Macaques R04-016 and R06-015 received F(-)SeV-Gag216S IN and F(-)SeV-Gag$_{202-216}$-EGFP IM at the second vaccination and vice versa at the third.

(BD, Tokyo, Japan) and the following monoclonal antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-human CD4 (BD, #556615, M-T477), peridinin chlorophyll protein (PerCP)-conjugated anti-human CD8 (BD, #347314, SK1), allophycocyanin (APC)-conjugated anti-human CD3 (BD, #557597, SP34-2), and phycoerythrin (PE)-conjugated anti-human IFN-γ antibodies (BD, #557074, 4S.B3). Specific CD8$^+$ T-cell levels were calculated by subtracting non-specific IFN-γ$^+$ CD8$^+$ T-cell frequencies from those after Gag epitope peptide-specific or SeV-specific stimulation. Specific CD8$^+$ T-cell levels lower than 100 per million PBMCs were considered negative.

2.4. Measurement of anti-SeV IgG levels

The plasma anti-SeV immunoglobulin G (IgG) levels were measured by an enzyme-linked immunosorbent assay (ELISA) (Denka Seiken, Tokyo, Japan) using whole inactivated SeV (HVJ Z strain) particles and a peroxidase-conjugated anti-monkey IgG antibody [30].

2.5. Measurement of anti-SeV neutralizing titers

We measured plasma SeV-specific neutralizing titers on LLC-MK2 cells using a recombinant SeV expressing enhanced

green fluorescent protein (SeV-EGFP) as described before [31]. We determined the end-point plasma titers required for 10-fold reduction of SeV-EGFP infectivity compared to the negative control without plasma (90% neutralization titer; 90% effective concentration [EC_{90}]).

3. Results

3.1. Antigen-specific CD8$^+$ T-cell responses after repeated F(-)SeV vector vaccination

We used four SIV controllers possessing the MHC-I haplotype *90-120-Ia* [22,24]. In these rhesus macaques that were vaccinated and challenged with SIV in our previous studies [25,27], plasma viremia was undetectable in the chronic phase. In the present study, these four animals received SeV vector vaccination three times with intervals of three weeks in the chronic phase. Three kinds of SeV vectors, F(-)SeV-Gag inducing both $Gag_{206-216}$ epitope-specific and $Gag_{241-249}$ epitope-specific CD8$^+$ T-cell responses, F(-)SeV-$Gag_{202-216}$-EGFP inducing the former, and F(-)SeV-Gag216S inducing the latter, were used for the vaccination (Fig. 1). All four macaques received an intranasal F(-)SeV-Gag vector inoculation at the first vaccination. Macaques R04-015 and R06-035 received F(-)SeV-$Gag_{202-216}$-EGFP intranasally and F(-)SeV-Gag216S intramuscularly at the second vaccination and vice versa at the third. Macaques R04-016 and R06-015 received F(-)SeV-Gag216S intranasally and F(-)SeV-$Gag_{202-216}$-EGFP intramuscularly at the second vaccination and vice versa at the third.

Previously, macaques possessing the MHC-I haplotype *90-120-Ia* were shown to induce $Gag_{206-216}$ and $Gag_{241-249}$ epitope-specific CD8$^+$ T-cell responses dominantly in the early phase after SIVmac239 challenge [25,27]. In the present study, we examined these $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CD8$^+$ T-cell responses one week after each vaccination (Fig. 2). The first F(-)SeV-Gag vaccination enhanced both $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CD8$^+$ T-cell responses in macaques R06-035 and R06-015 but not in R04-016. In macaque R04-015, efficient $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CD8$^+$ T-cell responses were detected at week 1 after the first vaccination, while PBMC samples were unavailable for analysis of responses just before the first vaccination.

At week 4, one week after the second vaccination, animals had similar or higher $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CD8$^+$ T-cell frequencies compared to those at week 1. Also at week 7, one week after the third vaccination, animals had similar or higher $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CD8$^+$ T-cell frequencies compared to those at week 4. Enhancement of these Gag-specific CD8$^+$ T-cell response was clear after the second vaccination (at week 4) in macaques R04-016 and R06-035 and after the third vaccination (at week 7) in macaques R04-015 and R06-015. Thus, all four animals showed higher Gag-specific CD8$^+$ T-cell responses at week 7 compared to those at week 1, indicating enhancement of Gag-specific CD8$^+$ T-cell responses by the second/third vaccination.

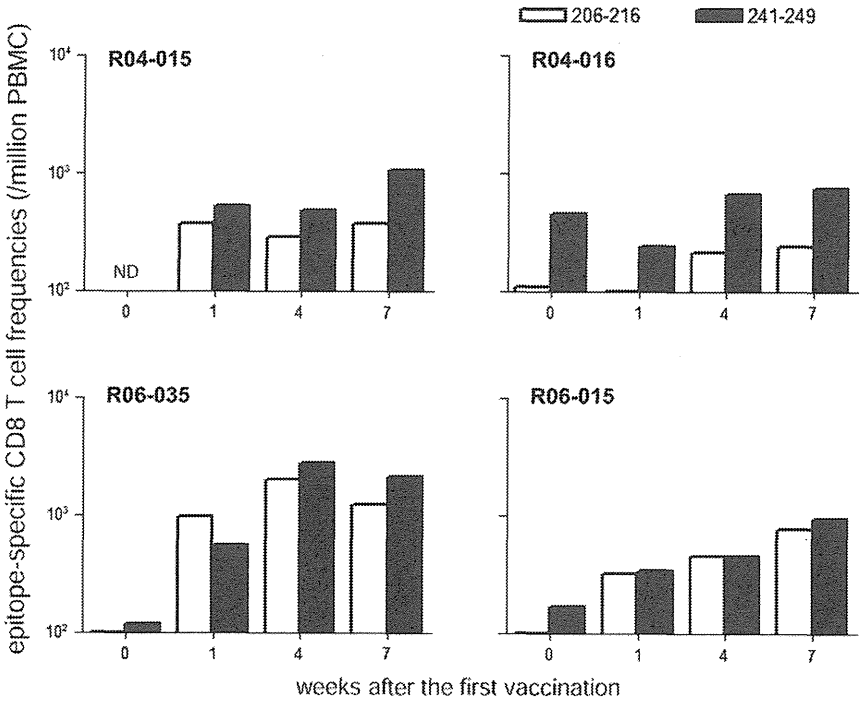


Fig. 2. Gag epitope-specific CD8$^+$ T-cell responses after repeated SeV vector vaccination. $Gag_{206-216}$-specific (open boxes) and $Gag_{241-249}$-specific (closed boxes) CD8$^+$ T-cell responses at week 0 (just before the first vaccination) and at weeks 1, 4, and 7 after the first vaccination (one week after each vaccination) were examined. ND, not determined.

3.2. SeV-specific CD8$^+$ T-cell responses after repeated F(-)SeV vector vaccination

We also examined SeV-specific CD8$^+$ T-cell responses one week after each vaccination (Fig. 3). SeV-specific CD8$^+$ T-cell responses were undetectable at week 0, just before the first vaccination. Vaccination mostly enhanced SeV-specific CD8$^+$ T-cell responses, and all animals showed efficient SeV-specific CD8$^+$ T-cell responses after three times of vaccination. Clear difference in the patterns of enhancement was not observed between $Gag_{206-216}$/$Gag_{241-249}$-specific and SeV-specific CD8$^+$ T-cell responses, suggesting little or no change in the immunodominance patterns between these CD8$^+$ T-cell responses by the second/third SeV vector vaccination.

3.3. SeV-specific antibody responses at repeated F(-)SeV vector vaccination

We then examined SeV-specific antibody responses just before each vaccination (Fig. 4 and Fig. 5). While these animals received a single SeV vector vaccination in previous studies more than a year before, ELIZA showed marginal levels of SeV-specific antibodies at week 0, just before the first vaccination. These animals exhibited undetectable or low levels of SeV-specific neutralizing antibody responses at week 0. At weeks 3 and 6, just before the second/third vaccination, all four animals had high levels of SeV-specific IgGs and neutralizing titers, indicating that the second/third SeV vector vaccination enhanced Gag-specific CD8$^+$ T-cell responses in the presence of high levels of SeV-specific neutralizing responses.

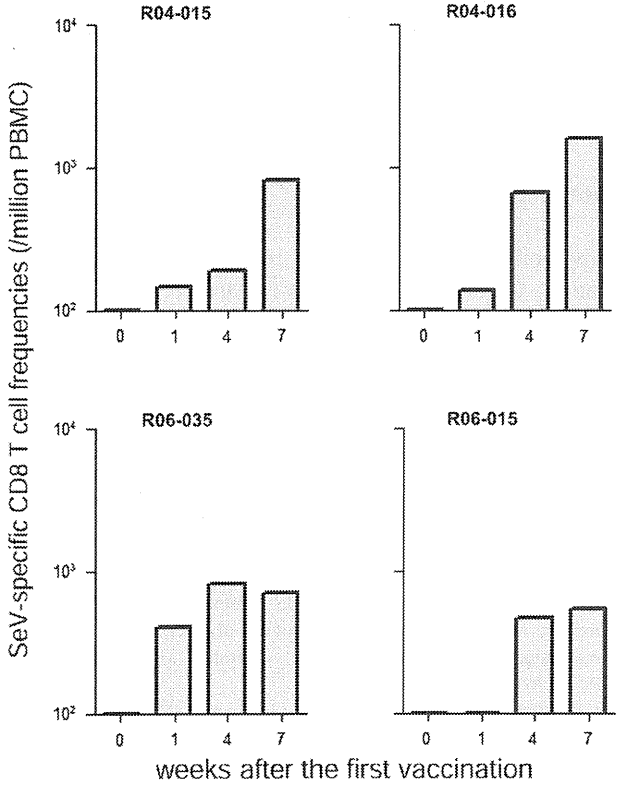


Fig. 3. SeV-specific CD8$^+$ T-cell responses after repeated SeV vector vaccination. SeV-specific CD8$^+$ T-cell responses at week 0 (just before the first vaccination) and at weeks 1, 4, and 7 after the first vaccination (one week after each vaccination) were examined.

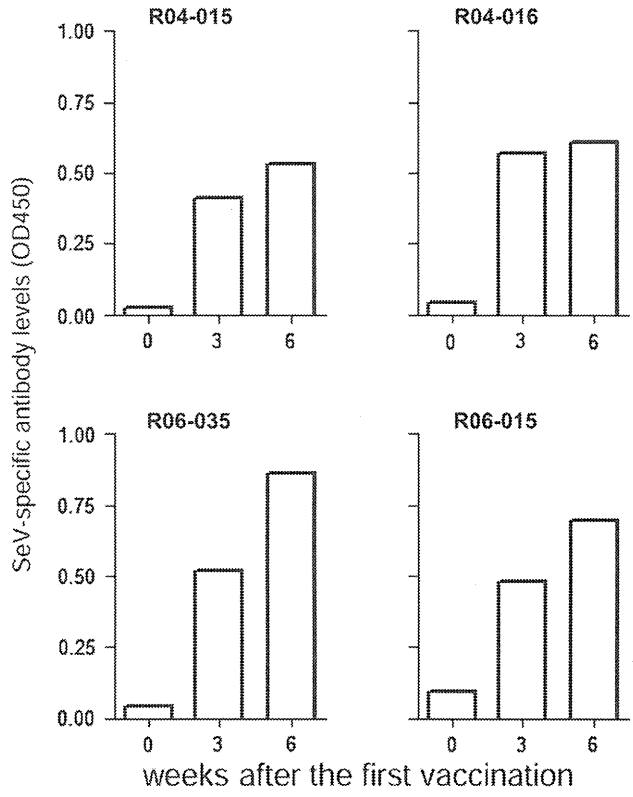


Fig. 4. SeV-specific IgG levels in plasma at repeated SeV vector vaccination. Plasma samples obtained at weeks 0, 3, and 6 after the first vaccination (just before each vaccination) were subjected to ELISA assay. OD450, optical density at 450 nm.

4. Discussion

Replication-defective viral vectors are promising safe tools for eliciting antigen-specific T-cell responses. A single inoculation of these vectors can induce efficient T-cell responses, which would peak within a couple of weeks and decline after that, although durable T-cell memory induction is important for vaccine efficacy. In the present study, we examined immunogenicity of three times of replication-defective SeV vector vaccination with intervals of three weeks in macaques and showed that antigen-specific CD8$^+$ T-cell responses after the third vaccination were higher than those after the first vaccination. Our results indicate the potential of repeated SeV vector vaccination to induce efficient and durable T-cell responses, providing a solution toward durable vaccine efficacy.

CD8$^+$ T-cells recognize MHC-I-restricted epitopes presented on target cells, and animals sharing MHC-I alleles would be useful for exact evaluation of vaccine immunogenicity. We confirmed $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CD8$^+$ T-cell responses in macaques R04-015, R04-

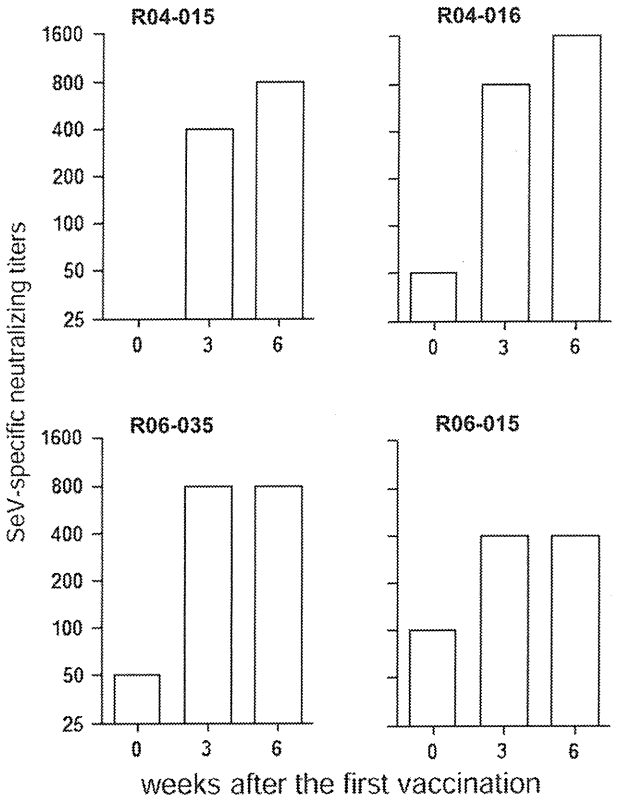


Fig. 5. SeV-specific neutralizing titers in plasma at repeated SeV vector vaccination. Plasma samples obtained at weeks 0, 3, and 6 after the first vaccination (just before each vaccination) were subjected to anti-SeV neutralizing assay. The end-point plasma titers required for 10-fold reduction of SeV-EGFP infectivity are shown.

016, R06-015, and R06-035 sharing MHC-I haplotype *90-120-Ia* after SIVmac239 challenge in our previous studies [25–27]. Then, we have focused on analyzing $Gag_{206-216}$-specific and $Gag_{241-249}$-specific $CD8^+$ T-cell responses for evaluation of vaccine immunogenicity in the present study.

Previous studies have shown that Gag-specific $CD8^+$ T-cell frequencies peaked around one week after a single F(-)SeV-Gag vaccination [19]. Indeed, Gag-specific $CD8^+$ T-cell responses became undetectable in a few months in five of six F(-)SeV-Gag-boosted macaques [32]. Then, it is inferred that $Gag_{206-216}$-specific and $Gag_{241-249}$-specific $CD8^+$ T-cell frequencies would be much lower at weeks 4 and 7 than those at week 1 without repeated vaccination. In the present study, however, those frequencies at week 7 were higher than at week 1. $Gag_{206-216}$-specific and $Gag_{241-249}$-specific $CD8^+$ T-cell frequencies at weeks 4 and 7 were similar or rather higher than those at weeks 1 and 4, respectively. These results imply that each of the second and the third vaccination enhanced these $CD8^+$ T-cell responses, leading to augmented, durable $Gag_{206-216}$-specific and $Gag_{241-249}$-specific $CD8^+$ T-cell responses.

Pre-existing anti-vector antibodies can be an obstacle to the immunogenicity of viral vectors [15,33–35]. Viral vector vaccination induces immune responses against the vector virus itself, and so, anti-vector antibodies could inhibit induction of antigen-specific T-cell responses by repeated vaccination. Indeed, after the first vaccination, all four animals had high levels of SeV-specific neutralizing antibodies, which may have affected efficacy of T-cell induction by the second and the third SeV vector vaccination with short intervals. However, the second and the third SeV vector vaccination in the presence of high levels of SeV-specific neutralizing antibodies enhanced $Gag_{206-216}$-specific and $Gag_{241-249}$-specific $CD8^+$ T-cell responses.

Recently, we showed that intranasal SeV vector immunization is more immunogenic than intramuscular in the presence of SeV-specific neutralizing antibodies [20]. In that study, Gag-specific $CD8^+$ T-cell responses were induced not by intramuscular but by intranasal immunization with 6×10^8 CIU of F(-)SeV-Gag vectors in the presence of 1:100 of plasma SeV-specific neutralizing titers. We were unable to quantify SeV-specific IgA levels. In the present study, however, clear difference in immunogenicity was not shown between intranasal and intramuscular SeV vector vaccination. Enhancement of antigen-specific $CD8^+$ T-cell responses by the second, intramuscular vaccination was observed in macaques R04-016 and R06-035 (Fig. 2). Intramuscular immunization with higher doses (6×10^9 CIU) may overcome the inhibitory effect by SeV-specific neutralizing antibodies.

In summary, we examined antigen-specific $CD8^+$ T-cell responses in macaques after three times of SeV vector vaccination with intervals of three weeks. Antigen-specific $CD8^+$ T-cell responses did not decline but were enhanced by the second and the third vaccination even in the presence of high levels of SeV-specific neutralizing antibodies. These results indicate that repeated SeV vector vaccination even with short intervals can contribute to induction of efficient, durable T-cell responses.

Acknowledgments

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A Novel Protective MHC-I Haplotype Not Associated with Dominant Gag-Specific CD8$^+$ T-Cell Responses in SIVmac239 Infection of Burmese Rhesus Macaques

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Abstract

Several major histocompatibility complex class I (MHC-I) alleles are associated with lower viral loads and slower disease progression in human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) infections. Immune-correlates analyses in these MHC-I-related HIV/SIV controllers would lead to elucidation of the mechanism for viral control. Viral control associated with some protective MHC-I alleles is attributed to CD8$^+$ T-cell responses targeting Gag epitopes. We have been trying to know the mechanism of SIV control in multiple groups of Burmese rhesus macaques sharing MHC-I genotypes at the haplotype level. Here, we found a protective MHC-I haplotype, *90-010-Id* (D), which is not associated with dominant Gag-specific CD8$^+$ T-cell responses. Viral loads in five D$^+$ animals became significantly lower than those in our previous cohorts after 6 months. Most D$^+$ animals showed predominant Nef-specific but not Gag-specific CD8$^+$ T-cell responses after SIV challenge. Further analyses suggested two Nef-epitope-specific CD8$^+$ T-cell responses exerting strong suppressive pressure on SIV replication. Another set of five D$^+$ animals that received a prophylactic vaccine using a Gag-expressing Sendai virus vector showed significantly reduced viral loads compared to unvaccinated D$^+$ animals at 3 months, suggesting rapid SIV control by Gag-specific CD8$^+$ T-cell responses in addition to Nef-specific ones. These results present a pattern of SIV control with involvement of non-Gag antigen-specific CD8$^+$ T-cell responses.

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Introduction

Virus-specific CD8$^+$ T-cell responses play a central role in the control of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) replication [1,2,3,4,5]. Genetic diversities of HLA or major histocompatibility complex class I (MHC-I) result in various patterns of CD8$^+$ T-cell responses in HIV-infected individuals. Cumulative studies on HIV infection have indicated the association of MHC-I genotypes with higher or lower viral loads [6,7,8,9,10]. In some MHC-1 alleles associating with lower viral loads and slower disease progression, certain CD8$^+$ T-cell responses restricted by these MHC-I molecules have been shown to be responsible for HIV control [11,12,13]. In rhesus macaque AIDS models, *Mamu-A*01*, *Mamu-B*08*, and *Mamu-B*17* are known as protective alleles, and macaques possessing these alleles tend to show slower disease progression after SIVmac251/SIVmac239 challenge [14,15,16,17].

Recent studies have indicated great contribution of CD8$^+$ T-cell responses targeting Gag epitopes to reduction in viral loads in HIV/SIV infection [18,19,20,21]. Viral control associated with some protective MHC-I alleles is attributed to Gag epitope-specific CD8$^+$ T-cell responses [22,23,24]. For instance, CD8$^+$ T-cell responses specific for the HLA-B*57-restricted $Gag_{240-249}$ TW10 and HLA-B*27-restricted $Gag_{263-272}$ KK10 epitopes exert strong suppressive pressure on HIV replication and frequently select for an escape mutation with viral fitness costs, leading to lower viral loads [22,24,25,26,27]. On the other hand, CD8$^+$ T-cell responses targeting SIV antigens other than Gag, such as Mamu-B*08- or Mamu-B*17-restricted Vif and Nef epitopes, have been indicated to exert strong suppressive pressure on SIV replication [28,29,30,31,32,33]. Accumulation of our knowledge on the potential of these non-Gag-specific as well as Gag-specific CD8$^+$ T-cell responses for HIV/SIV control should be encouraged for elucidation of viral control mechanisms.

We have been examining SIVmac239 infection in multiple groups of Burmese rhesus macaques sharing MHC-I genotypes at the haplotype level and indicated an association of MHC-I haplotypes with AIDS progression [21,34]. In our previous study, a group of macaques sharing MHC-I haplotype *90-120-Ia* (A) induced dominant Gag-specific $CD8^+$ T-cell responses and tended to show slower disease progression after SIVmac239 challenge [21]. Prophylactic immunization of these A^+ macaques with a DNA vaccine prime and a Gag-expressing Sendai virus (SeV-Gag) vector boost resulted in SIV control based on Gag-specific $CD8^+$

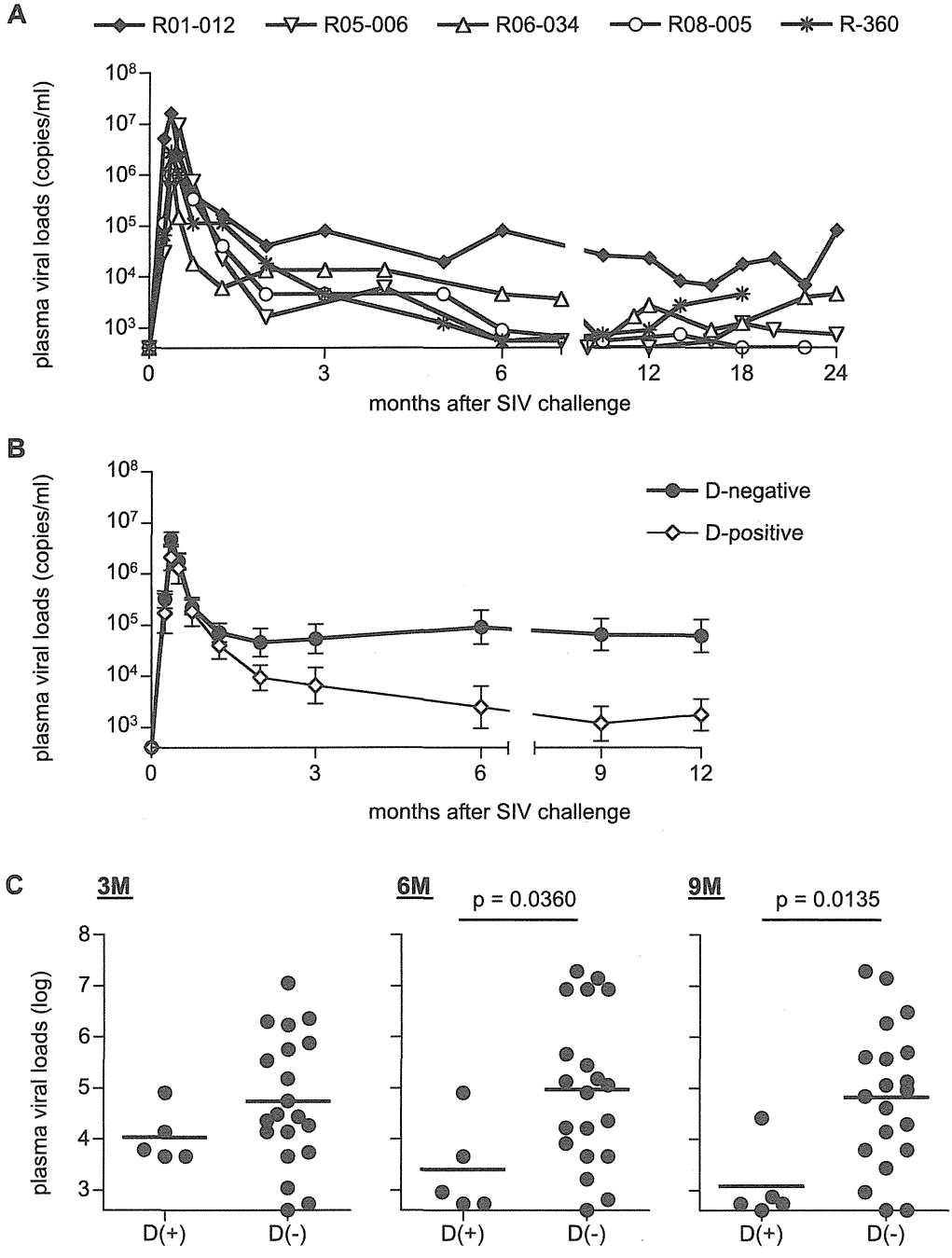

Figure 1. Plasma viral loads after SIVmac239 challenge in unvaccinated macaques. Plasma viral loads (SIV *gag* RNA copies/ml plasma) were determined as described previously [35]. The lower limit of detection is approximately 4×10^2 copies/ml. (A) Changes in plasma viral loads after challenge in unvaccinated macaques possessing MHC-I haplotype D. (B) Changes in geometric means of plasma viral loads after challenge in five unvaccinated D^+ animals in the present study and twenty D^- animals in our previous cohorts [21]. Three of twenty D^- animals were euthanized because of AIDS before 12 months, and we compared viral loads between D^+ and D^- animals until 12 months. (C) Comparison of plasma viral loads at 3 months (left panel), 6 months (middle panel), and 9 months (right panel) between the unvaccinated D^+ and the D^- animals. Viral loads at 6 months and 9 months in D^+ animals were significantly lower than those in the latter D^- animals ($p=0.0360$ at 6 months and $p=0.0135$ at 9 months by t-test).
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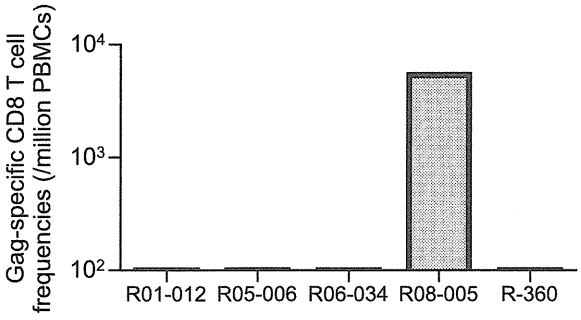

Figure 2. SIV Gag-specific CD8$^+$ T-cell responses in unvaccinated D$^+$ macaques at week 2 after SIVmac239 challenge.
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T-cell responses [35,36]. Accumulation of data on interaction between virus replication and T-cell responses in multiple groups of macaques sharing individual MHC-I haplotypes would provide great insights into our understanding of the mechanism for HIV/SIV control.

In the present study, we investigated SIVmac239 infection of a group of Burmese rhesus macaques possessing the MHC-I haplotype *90-010-Id* (D), which was not associated with dominant Gag-specific CD8$^+$ T-cell responses. These animals had persistent viremia in the early phase but showed significant reduction of viral loads around 6 months after SIV challenge. Most D$^+$ animals showed predominant Nef-specific but not Gag-specific CD8$^+$ T-cell responses. This study presents a protective MHC-I haplotype, indicating the potential of non-Gag antigen-specific CD8$^+$ T-cell responses to contribute to SIV control.

Materials and Methods

Ethics Statement

Animal experiments were carried out in National Institute of Biomedical Innovation (NIBP) and Institute for Virus Research in Kyoto University (IVRKU) after approval by the Committee on the Ethics of Animal Experiments of NIBP and IVRKU in accordance with the guidelines for animal experiments at NIBP, IVRKU, and National Institute of Infectious Diseases. To prevent viral transmission, animals were housed in individual cages allowing them to make sight and sound contact with one another, where the temperature was kept at 25°C with light in 12 hours per day. Animals were fed with apples and commercial monkey diet (Type CMK-2, Clea Japan, Inc. Tokyo). Blood collection, vaccination, and SIV challenge were performed under ketamine anesthesia. The endpoint for euthanasia was determined by typical signs of AIDS including reduction in peripheral CD4$^+$ T-cell counts (less than 200 cells/μl), 10% loss of body weight, diarrhea, and general weakness. At euthanasia, animals were deeply anesthetized with pentobarbital under ketamine anesthesia, and then, whole blood was collected from left ventricle.

Animal Experiments

We examined SIV infections in a group of Burmese rhesus macaques (n = 10) sharing the MHC-I haplotype *90-010-Id* (D). The determination of MHC-I haplotypes was based on the family study in combination with the reference strand-mediated conformation analysis (RSCA) of *Mamu-A* and *Mamu-B* genes and detection of major *Mamu-A* and *Mamu-B* alleles by cloning the reverse transcription (RT)-PCR products as described previously [21,34,37]. Macaques R01-012 and R01-009 used in our previous report [35] and macaques R03-021 and R03-016 used in an unpublished experiment were included in the present study. Five macaques R01-009, R06-020, R06-033, R03-021, and R03-016 received a prophylactic DNA prime/SeV-Gag boost vaccine (referred to as DNA/SeV-Gag vaccine) [35]. The DNA used for the vaccination, CMV-SHIVdEN, was constructed from an *env*-deleted and *nef*-deleted simian–human immunodeficiency virus SHIVMD14YE [38] molecular clone DNA (SIVGP1) and has the genes encoding SIVmac239 Gag, Pol, Vif, and Vpx, and HIV Tat and Rev. At the DNA vaccination, animals received 5 mg of CMV-SHIVdEN DNA intramuscularly. Six weeks after the DNA prime, animals received a single boost intranasally with 6×10^9 cell infectious units (CIUs) of F-deleted replication-defective SeV-Gag [39,40]. All animals were challenged intravenously with 1,000 $TCID_{50}$ (50 percent tissue culture infective doses) of SIVmac239 [41]. At week 1 after SIV challenge, macaque R03-021 was inoculated with nonspecific immunoglobulin G (IgG) and macaques R03-016 with IgG purified from neutralizing antibody-positive plasma of chronically SIV-infected macaques in our previous experiment [42].

Analysis of SIV Antigen-specific CD8$^+$ T-cell Responses

SIV antigen-specific CD8$^+$ T-cell responses were measured by flow-cytometric analysis of gamma interferon (IFN-γ) induction as described previously [43]. Autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines (B-LCLs) were established from peripheral blood mononuclear cells (PBMCs) which were obtained from individual macaques before SIV challenge [44]. PBMCs obtained from SIV-infected macaques were cocultured with autologous B-LCLs pulsed with peptides or peptide pools using panels of overlapping peptides spanning the entire SIVmac239 Gag, Pol, Vif, Vpx, Vpr, Tat, Rev, Env, and Nef amino acid sequences. Alternatively, PBMCs were cocultured with B-LCLs infected with a vaccinia virus vector expressing SIVmac239 Gag for Gag-specific stimulation. Intracellular IFN-γ staining was performed using CytofixCytoperm kit (BD, Tokyo, Japan). Fluorescein isothiocianate-conjugated anti-human CD4 (BD), Peridinin chlorophyll protein (PerCP)-conjugated anti-human CD8 (BD), allophycocyanin Cy7 (APC-Cy7)-conjugated anti-human CD3 (BD), and phycoerythrin (PE)-conjugated anti-human IFN-γ antibodies (Biolegend, San Diego, CA) were used. Specific T-cell levels were calculated by subtracting non-specific IFN-γ$^+$ T-cell frequencies from those after peptide-specific stimulation. Specific T-cell levels less than 100 cells per million PBMCs were considered negative.

Sequencing Analysis of Plasma Viral Genomes

Viral RNAs were extracted using High Pure Viral RNA kit (Roche Diagnostics, Tokyo, Japan) from macaque plasma samples. Fragments of cDNAs encoding SIVmac239 Gag and Nef were amplified by nested RT-PCR from plasma RNAs and subjected to direct sequencing by using dye terminator chemistry and an automated DNA sequencer (Applied Biosystems, Tokyo, Japan) as described before [45]. Predominant non-synonymous mutations were determined.

Statistical Analysis

Statistical analysis was performed using Prism software version 4.03 with significance levels set at a P value of <0.050 (GraphPad Software, Inc., San Diego, CA). Plasma viral loads were log transformed and compared by an unpaired two-tailed t test.

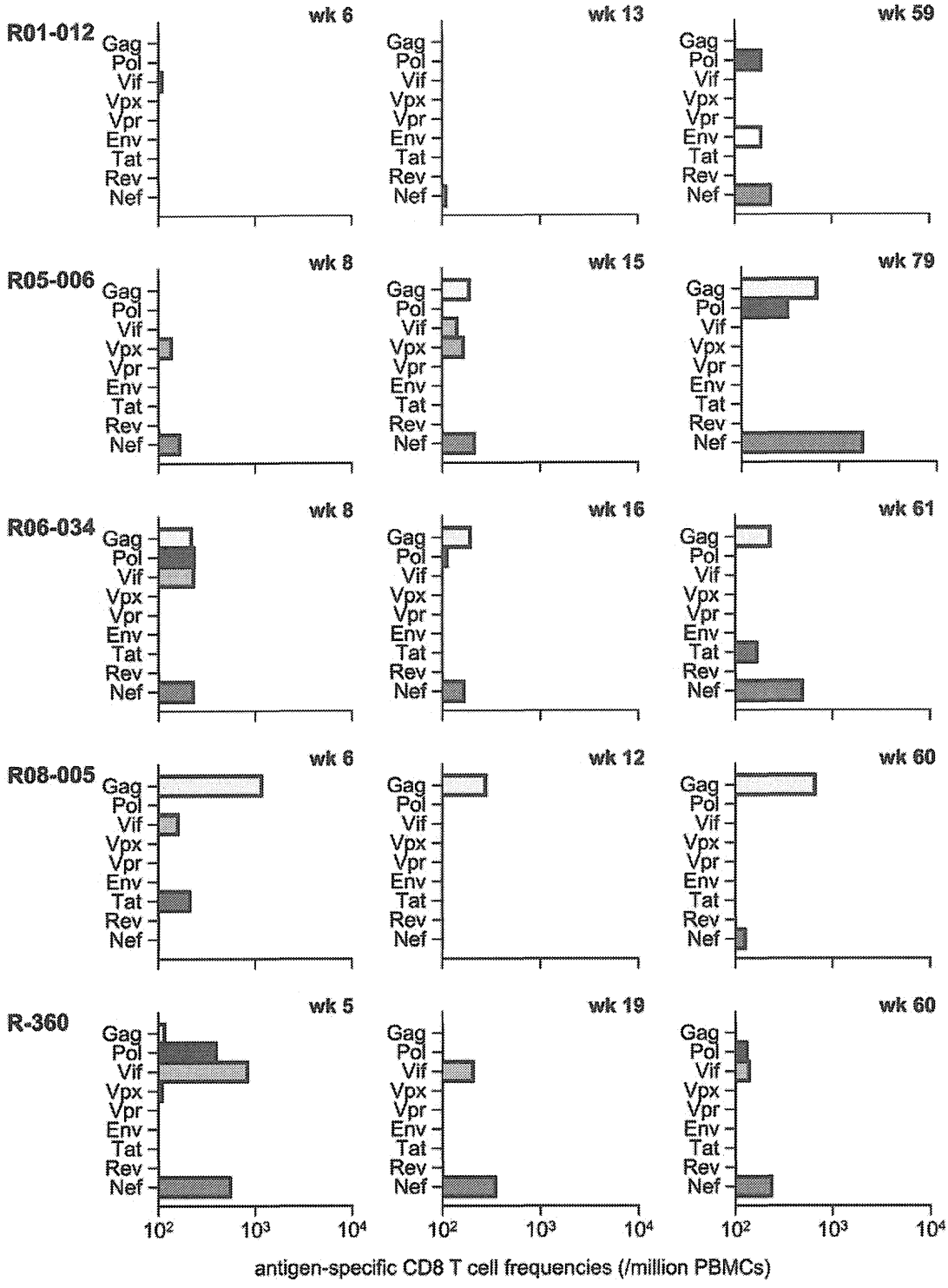

Figure 3. SIV antigen-specific CD8$^+$ T-cell responses in unvaccinated D$^+$ macaques. Responses were measured by the detection of antigen-specific IFN-γ induction in PBMCs obtained at indicated time points after SIVmac239 challenge.
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Results

Lower Viral Loads in D$^+$ Macaques in the Chronic Phase of SIV Infection

We first investigated SIVmac239 infection of five unvaccinated Burmese rhesus macaques sharing the MHC-I haplotype D (referred to as D$^+$ macaques). Confirmed MHC-I alleles consisting of this haplotype is *Mamu-A1*032:02*, *Mamu-B*004:01*, and *Mamu-B*102:01:01*. These animals showed lower set-point plasma viral loads (Fig. 1). Comparison of plasma viral loads between these five animals and our previous cohorts of SIVmac239-infected Burmese D-negative (D$^-$) rhesus macaques (n = 20) [21] revealed no