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サル免疫不全ウイルス中和抗体の感染個体レベルにおける防御機序の解析

平成２３年度　総括研究報告書

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目次

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研究要旨

エイズウイルス（ヒト免疫不全ウイルス、HIV）感染症の最大の問題は、自然感染経過において T 細胞応答不全と中和抗体（NAb）反応の欠失を伴い慢性持続感染化する事であるが、その成立過程は明らかではない。これに対し研究代表者は近年、SIV（サル免疫不全ウイルス）感染サルエイズモデルにおいてピーク体内感染期の中和抗体受動免疫による特異的 T 細胞応答亢進を伴った顕著な持続感染阻止効果を証明し、機序として樹状細胞（DC）への Fc 依存性中和抗体-ウイルス粒子複合体取込み促進による抗原提示亢進が関わりうる可能性を見出した。本研究では、(1)「SIV 中和抗体による抗原提示亢進を介し感染後新規に T 細胞を誘導する」機構に関わる DC 受容体を同定し、(2) SIV 感染-中和抗体受動免疫サルエイズモデルにおいてその阻害実験を行い中和抗体の個体レベルの作用機序を網羅的に解明し、(3) 中和抗体存在下で誘導される特異的 T 細胞の質を試験管内・個体レベルで評価を行い、液性・細胞性免疫による相乗的なエイズウイルス防御機構の可能性評価を目標とした。1 年目は NAb の抗原提示能に関わる DC 受容体として CD64 を見出し、より重要な知見として NAb 存在下で CD8 陽性 T 細胞の特異的 CCL4 産生亢進を認めた。2 年目はまず SIV 感染・NAb 受動免疫時の CD64 阻害予備実験を行い、さらに前年度の抗体による CD8 直接誘導の知見に基づき抗体の中和能の必要性を検証することを最重要視し、①非・中和抗体（nNAb）の大量精製を行い、②ELISA 法のスクリーニング系を立ち上げ、③それに基づき背景研究で使用した NAb と同じ粒子結合能を有する nNAb を選抜して、④SIV 感染急性期の受動免疫実験を開始した。本研究の成果は、SIV 感染初期の中和抗体受動免疫時におけるウイルス複製制御の必要十分条件の解明に結び付くものであり、中和抗体誘導型予防エイズワクチン開発への論理的基盤に寄与することが期待される。

A.　研究目的

本研究は、エイズウイルス（ヒト免疫不全ウイルス、HIV）の感染個体レベルにおける防御に極めて重要なウイルス中和抗体につき、その作用機序の全容をサルエイズモデルを用いて網羅的に明らかとし、それにより予防エイズワクチン開発への論理的基盤を見出すことを目的としている。

エイズウイルス感染症の制御において特異的 T 細胞

応答の重要性は以前より指摘されてきたのに対し、中和抗体を代表とする液性免疫応答の位置付けは明らかとなっていなかった。これに対し研究代表者は近年SIV感染サルエイズモデルにおいて、感染初期の中和抗体受動免疫により特異的T細胞の誘導亢進が生じ顕著な持続感染成立阻止効果が呈される事を初めて証明した（*PLoS ONE* 2: e540, 2007; *J Virol.* 83: 5514-5524, 2009）。本研究はその結果を踏まえ、特にその機序として樹状細胞への中和抗体-ウイルス粒子複合体取込み・抗原提示亢進がどの程度関わるかを中心として中和抗体の個体レベル防御機構の全容解明を期する。即ち当該研究は「抗エイズウイルス中和抗体を介したT細胞の誘導」が予防ワクチンによるHIV制御の全く新しい防御戦略となる可能性を検証する点で、非常に重要性が高いと考えられる。検証を試みる「抗ウイルス液性免疫を介したT細胞誘導」は、後者→前者の影響の解析に限られてきた両者間で、樹状細胞を介し前者→後者の顕著な感染時の修飾が生じ、更にその積極動員で元来困難なHIV制御が達されうる可能性を初めて提起する概念である。本研究により、エイズウイルス中和抗体の限定的な機序として過去に提唱された補体活性化、ADCCなどの作用で説明を充足し得ない個体レベルの防御機構の網羅的な解明が初めて可能となることが考えられる。抗体-病原体複合体の単球系細胞による貪食作用は細菌・寄生虫感染で解析されてきたが、①ウイルス感染で、更に②その排除がT細胞に専ら依存すると捉えられる持続感染症の制御の成否に中和抗体が関わり、樹状細胞への抗原提示がその中心的機序である可能性を検証するのは本研究が初めてであり、感染症基礎研究としての理学的観点、新規エイズ制御戦略という工学的観点のいずれにおいても重要な基礎情報を提供することが期待される。

平成２２年度は、中和抗体を介したSIV抗原提示亢進を司る樹状細胞受容体の検索および中和抗体-SIV複合体パルス樹状細胞により誘導される細胞性免疫の解析を行った。これに基づき、平成２３年度は主に以下の検討を行った。

１：SIV感染初期のNAb受動免疫時におけるCD64阻害抗体の共接種予備実験

２：NAbによるSIV制御時の中和能の必要性評価

B. 研究方法

１：SIV感染初期のNAb受動免疫時におけるCD64阻害抗体の共接種予備実験

アカゲサル群にSIVを接種後７日目にNAb受動免疫した際CD64結合抗体を共接種し、血中ウイルス量とNAb受動免疫直後のDC分画SIVゲノム数、リンパ節中SIV p27陰性CCR7/CD11c陽性DCの流入率を評価した。

２：NAbによるSIV制御時の中和能の必要性評価：SIV感染時の抗SIV結合・非中和抗体受動免疫実験

抗原提示アッセイでNAbが感染標的とならない特異的CTLのCCL4産生亢進を誘導したことを重視し、この反応のみ個体内で起こすことを目的に「粒子結合（抗原提示）能を有し」、「中和能を有さない」SIV結合・非中和抗体（nNAb）の受動免疫実験を行うこととした。

①SIVmac239持続感染アカゲサル群をスクリーニングし、ウイルス中和活性を示さなかった個体の血漿よりポリクローナルIgGを精製した。

②感染性SIV粒子を抗原としたELISA系を樹立した。サルT細胞株（HSC-F）株にSIVを感染させ、上清中に産生されるウイルスをショ糖濃度勾配法により精製し、p27量換算で10ngを96穴プレートに固相化した。SIV感染あるいは非感染サルの血漿より精製したポリクローナル抗体（0.01～100 μg/ml）を試料とし、界面活性剤非存在下で間接酵素抗体法により各抗体のSIV粒子に対する結合性を評価した。

③上記の系に基づき、背景研究で使用したNAbと同様の粒子結合能を有するnNAbを選抜した。

④選抜したnNAbを用いたSIV感染急性期の受動免疫実験を開始し、抗体に中和能がなくCD8陽性T細胞誘導に偏りうる抗原提示で複製制御が得られるかを評価した。対照群に6頭、非中和抗体受動免疫群に5頭用いた。

（倫理面への配慮）

当該研究における遺伝子組み換え生物等を用いる実験については、必要に応じた国立感染症研究所の機関承認および文部科学大臣承認（第二種使用等核酸防止措置確認申請承認）を既に取得済みである。また、全ての動物実験は、倫理面および動物愛護問題の観点から、国立感染症研究所、医薬基盤研究所の動物実験委員会の審査を受け、その承認を得たのち、医薬基盤研究所霊長類医科学研究センターにおいて、動物実験委員会が定めたルールおよびガイドラインに沿って遂行している。

C. 研究結果

1：SIV 感染初期の NAb 受動免疫時における CD64 阻害抗体の共接種実験

セットポイント期血中ウイルス量は CD64 抗体共接種群で対照群より増加する示唆が得られ、NAb 受動免疫直後の DC 分画 SIV ゲノム数の減少及びリンパ節中 SIV p27 陰性 CCR7/CD11c 陽性ミエロイド DC の遊走率低下を認めた。後述する抗体の中和能の必要性評価を最重要と認め一旦解析を保留した。

2：NAb による SIV 制御時の中和能の必要性評価：確立した SIV 全粒子を抗原とする ELISA 法では、非感染個体由来の試料抗体（陰性対照群）は陰性であり、SIV 感染個体由来の試料抗体では結合性は陰性から高度陽性まで多様であった。評価した 10 頭由来のポリクローナル抗体の SIV 粒子に対する結合親和性評価に基づき、背景研究で使用した NAb と同じ粒子結合能を有する 3 頭由来の nNAb を選抜した。選抜した nNAb（合計 300mg）を SIV 感染急性期において受動免疫した結果、感染後 8 週の時点までては複製制御には至らない可能性が示唆されている。

D. 考察

SIVmac239 結合性・非中和抗体の新規スクリーニング・精製を通して新規動物実験系を樹立した結果、感染急性期の受動免疫抗体によるエイズウイルス持続感染成立阻止における中和能の必要性を示唆する知見が得られた。中和抗体受動免疫を行った先行研究においては①抗原提示能と②ウイルス中和能の寄与が両方考えられたが、このうち②に関する必要性が本段階で見出されたと考えられる。その理由としては、抗原取込みに続いて誘導される対象となりうる特異的CD4陽性T細胞の感染からの保護が不十分であると、有効な抗 SIV 細胞性免疫の誘導に至らない可能性が考えられうる。

中和能を有さないエイズウイルス結合抗体の果たしうる防御機序としては、①ウイルス粒子結合に引き続く抗原提示細胞への取込み、②感染細胞表面に残留する Env 蛋白への結合に引き続く抗体依存性細胞傷害（ADCC）等がこれまで報告されているが、本研究の結果はそれらのみでは持続感染成立の阻止に結び付かない可能性を示唆している。感染成立前の抗体受動免疫における感染成立阻止の条件としても ADCC に加え中和能の必要性が認められることが近年報告されており（Burton 2011）、本研究の結果はエイズウイルス特異抗体の感染成立前後で果たしうる防御機序を併せて描出するものである。次年度の課題としては、感染後 1 年以上の観察期間で非中和抗体受動免疫群におけるウイルス制御の長期予後を判定すること、及び感染急性期の特異的 CD8 陽性細胞の試験管内 SIV 複製抑制能、特異的 CD4 陽性 T 細胞の抗原特異的サイトカイン産生能などで病態を評価することが挙げられる。

E. 結論

アカゲサル群に SIV を接種後 7 日目に NAb 受動免疫した際に抗 CD64 抗体を共接種した結果、病態が悪化する可能性を認めた。本解析は一旦保留した。一方、前年度結果より更に重要な解析目標と認められた抗体の中和能の必要性評価のために「粒子結合（抗原提示）能を有し」、「中和能を有さない」SIV 結合・非中和抗体（nNAb）の受動免疫を計画した。nNAb の大量精製を行い、SIV 粒子 ELISA 法のスクリーニング系に基づき背景研究で使用した NAb と同じ粒子結合能を有する nNAb を選抜し、SIV 感染

急性期の受動免疫実験を開始し、初期感染後の制御に至らない可能性が示唆された。本研究の成果は、感染初期の抗体によるSIV持続感染阻止に必要十分な条件の解明に結び付くものであり、中和抗体誘導型予防エイズワクチン開発への論理的基盤に寄与することが期待される。

F. 健康危険情報

特になし。

G. 研究発表

1 論文発表

(1) Takahara Y, Matsuoka S, Kuwano T, Tsukamoto T, Yamamoto H, Ishii H, Nakasone T, Takeda A, Inoue M, Iida A, Hara H, Shu T, Hasegawa M, Sakawaki H, Horiike M, Miura T, Igarashi T, Naruse TK, Kimura A, Matano T. Dominant induction of vaccine antigen-specific cytotoxic T lymphocyte responses after simian immunodeficiency virus challenge.
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2 学会発表

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H. 知的財産権の出願・登録状況

1 特許登録

該当なし。

2 実用新案登録

該当なし。

3 その他

該当なし。

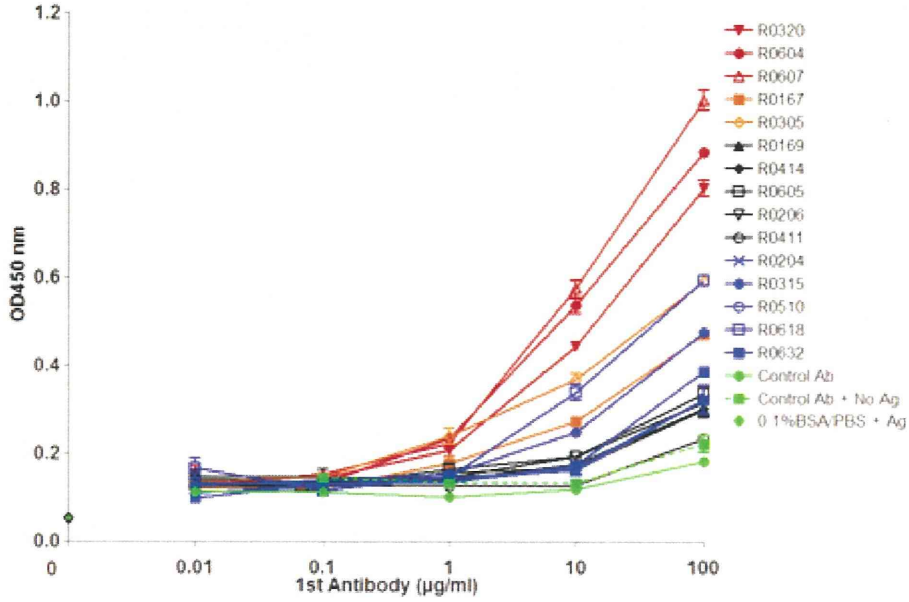
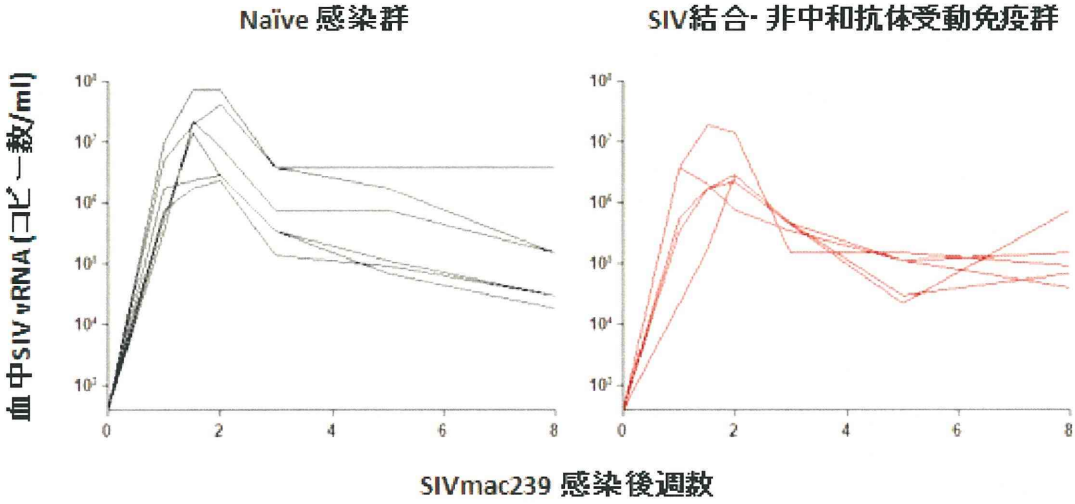


図１　ウイルス全粒子 ELISA 法を用いた SIV 結合・非中和抗体の結合能評価

感染性 SIV 粒子を抗原とした ELISA 系を確立した。サル T 細胞株（HSC-F）株に SIV を感染させ、上清中ウイルスをショ糖濃度勾配法により精製し、p27 量換算で 10ng を 96 穴プレートに固相化した。SIV 感染あるいは非感染サル血漿に由来するポリクローナル抗体（0.01～100 μg/ml）を試料とし、界面活性剤非存在下で間接酵素抗体法により各抗体の SIV 粒子に対する結合性を評価した。赤線は非中和抗体で高結合能、橙線は非中和抗体で中程度結合能、黒線は非中和抗体で低結合能を示した個体由来の検体を表す。青線は陽性対照としての各個体由来の中和抗体、緑線は陰性対照としての非感染個体由来コントロール抗体の結合を示す。

A



B

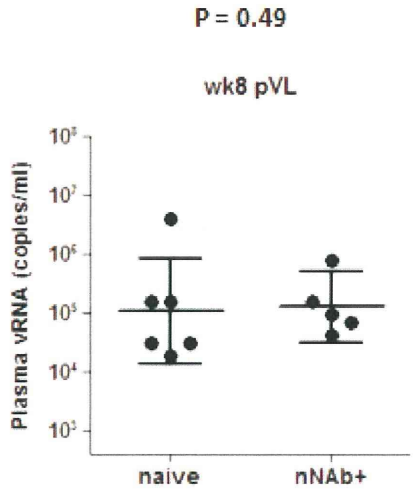


図２　アカゲサルに対する SIV 結合・非中和抗体の感染急性期における受動免疫実験

A.　アカゲサルに SIVmac239 をチャレンジしたのちの血漿中ウイルス量の感染初期における経時変化を示す。左（黒線）は対照群（n = 6）、右（赤線）は SIV 結合・非中和抗体受動免疫群（n = 5）を示す。横軸は感染後週数、縦軸は血中 SIV Gag RNA コピー数を表す。

B.　両群における感染後第８週の血中ウイルス量の比較を示す。Welch's unpaired t test で比較した結果、両群のウイルス量の差は有意でなかった（p = 0.49）。

研究成果の刊行に関する一覧表

書籍

該当なし。

雑誌

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Dominant induction of vaccine antigen-specific cytotoxic T lymphocyte responses after simian immunodeficiency virus challenge

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Immunodominance

ABSTRACT

Cytotoxic T lymphocyte (CTL) responses are crucial for the control of human and simian immunodeficiency virus (HIV and SIV) replication. A promising AIDS vaccine strategy is to induce CTL memory resulting in more effective CTL responses post-viral exposure compared to those in natural HIV infections. We previously developed a CTL-inducing vaccine and showed SIV control in some vaccinated rhesus macaques. These vaccine-based SIV controllers elicited vaccine antigen-specific CTL responses dominantly in the acute phase post-challenge. Here, we examined CTL responses post-challenge in those vaccinated animals that failed to control SIV replication. Unvaccinated rhesus macaques possessing the major histocompatibility complex class I haplotype *90-088-Ij* dominantly elicited SIV non-Gag antigen-specific CTL responses after SIV challenge, while those induced with Gag-specific CTL memory by prophylactic vaccination failed to control SIV replication with dominant Gag-specific CTL responses in the acute phase, indicating dominant induction of vaccine antigen-specific CTL responses post-challenge even in non-controllers. Further analysis suggested that prophylactic vaccination results in dominant induction of vaccine antigen-specific CTL responses post-viral exposure but delays SIV non-vaccine antigen-specific CTL responses. These results imply a significant influence of prophylactic vaccination on CTL immunodominance post-viral exposure, providing insights into antigen design in development of a CTL-inducing AIDS vaccine.

1. Introduction

In human and simian immunodeficiency virus (HIV and SIV) infections, cytotoxic T lymphocyte (CTL) responses exert strong suppressive pressure on viral replication but fail to control viremia leading to AIDS progression [1–5]. A promising AIDS vaccine strategy is to induce CTL memory resulting in more effective CTL responses post-viral exposure compared to those in natural HIV infections. It is important to determine how prophylactic CTL memory induction affects CTL responses in the acute phase post-viral exposure.

We previously developed a prophylactic AIDS vaccine (referred to as DNA/SeV-Gag vaccine) consisting of DNA priming followed by boosting with a recombinant Sendai virus (SeV) vector expressing SIVmac239 Gag [6]. Evaluation of this vaccine's efficacy against a SIVmac239 challenge in Burmese rhesus macaques showed that some vaccinees contained SIV replication [7]. In particular, vaccination consistently resulted in SIV control in those animals possessing the major histocompatibility complex class I (MHC-I) haplotype *90-120-Ia* [8]; $Gag_{206-216}$ (IINEEAADWDL) and $Gag_{241-249}$ (SSVDEQIQW) epitope-specific CTL responses were shown to be responsible for this vaccine-based SIV control [9]. Furthermore, in a SIVmac239 challenge experiment of *90-120-Ia*-positive macaques that received a prophylactic DNA/SeV vaccine expressing the $Gag_{241-249}$ epitope fused with enhanced green fluorescent protein (EGFP), all the vaccinees controlled SIV replication [10]. This single epitope vaccination resulted in dominant $Gag_{241-249}$-specific CTL responses with delayed $Gag_{206-216}$-specific CTL induction after SIV challenge, whereas $Gag_{206-216}$-specific and

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$Gag_{241-249}$-specific CTL responses were detected equivalently in unvaccinated *90-120-Ia*-positive animals.

These previous results in vaccine-based SIV controllers indicate dominant induction of vaccine antigen-specific CTL responses post-challenge, implying that prophylactic vaccination inducing vaccine antigen-specific CTL memory may delay CTL responses specific for viral antigens other than vaccine antigens (referred to as non-vaccine antigens) post-viral exposure. In these SIV controllers, the reduction of viral loads could be involved in delay of SIV non-vaccine antigen-specific CTL responses. Then, in the present study, we examined the influence of prophylactic vaccination on immunodominance post-challenge in those vaccinees that failed to control SIV replication. Our results showed dominant induction of vaccine antigen-specific CTL responses post-challenge even in these SIV non-controllers.

2. Materials and methods

2.1. Animal experiments

The first set of experiment used samples in our previous experiments of six Burmese rhesus macaques (Macaca mulatta) possessing the MHC-I haplotype *90-088-Ij* (macaques R02-004, R02-001, and R03-015, previously reported [7,11]; R04-014, R06-022, and R04-011, unpublished). Three of them, R02-001, R04-011, and R03-015, received a prophylactic DNA/SeV-Gag vaccine [7]. The DNA used for the vaccination, CMV-SHIVdEN, was constructed from *env*-deleted and *nef*-deleted simian–human immunodeficiency virus $SHIV_{MD14YE}$ [12] molecular clone DNA (SIVGP1) 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, animals received a single boost intranasally with 6×10^9 cell infectious units (CIUs) of F-deleted replication-defective SeV-Gag [13,14]. All six *90-088-Ij*-positive animals including three unvaccinated and three vaccinated were challenged intravenously with 1000 50% tissue culture infective doses (TCID50) of SIVmac239 [15] approximately 3 months after the boost. At week 1 after SIV challenge, macaque R03-015 was inoculated with nonspecific immunoglobulin G as previously described [11].

In the second set of experiment, unvaccinated (R06-001) and vaccinated (R05-028) rhesus macaques possessing the MHC-I haplotype *90-120-Ib* were challenged intravenously with 1000 TCID50 of SIVmac239. The latter R05-028 were immunized intranasally with F-deleted SeV-Gag approximately 3 months before the challenge.

In the third, three rhesus macaques received FMSIV plus mCAT1-expressing DNA vaccination three times with intervals of 4 weeks. The FMSIV DNA was constructed by replacing *nef*-deleted $SHIV_{MD14YE}$ with Friend murine leukemia virus (FMLV) *env*, carrying the same SIVmac239-derived antigen-coding regions with SIVGP1, as described before [16]. Vaccination of macaques with FMSIV and a DNA expressing the FMLV receptor (mCAT1) [17] three times with intervals of a week was previously shown to induce mCAT1-dependent confined FMSIV replication resulting in efficient CTL induction while vaccination three times with intervals of 4 weeks in the present study resulted in marginal levels of responses (data not shown). These three DNA-vaccinated animals were challenged intravenously with 1000 TCID50 of SIVmac239 approximately 2 months after the last vaccination.

Some animal experiments were conducted in the Tsukuba Primate Research Center, National Institute of Biomedical Innovation, with the help of the Corporation for Production and Research of Laboratory Primates, in accordance with the guidelines for animal experiments at the National Institute of Infectious Diseases, and others were in Institute for Virus Research, Kyoto University in accordance with the institutional regulations.

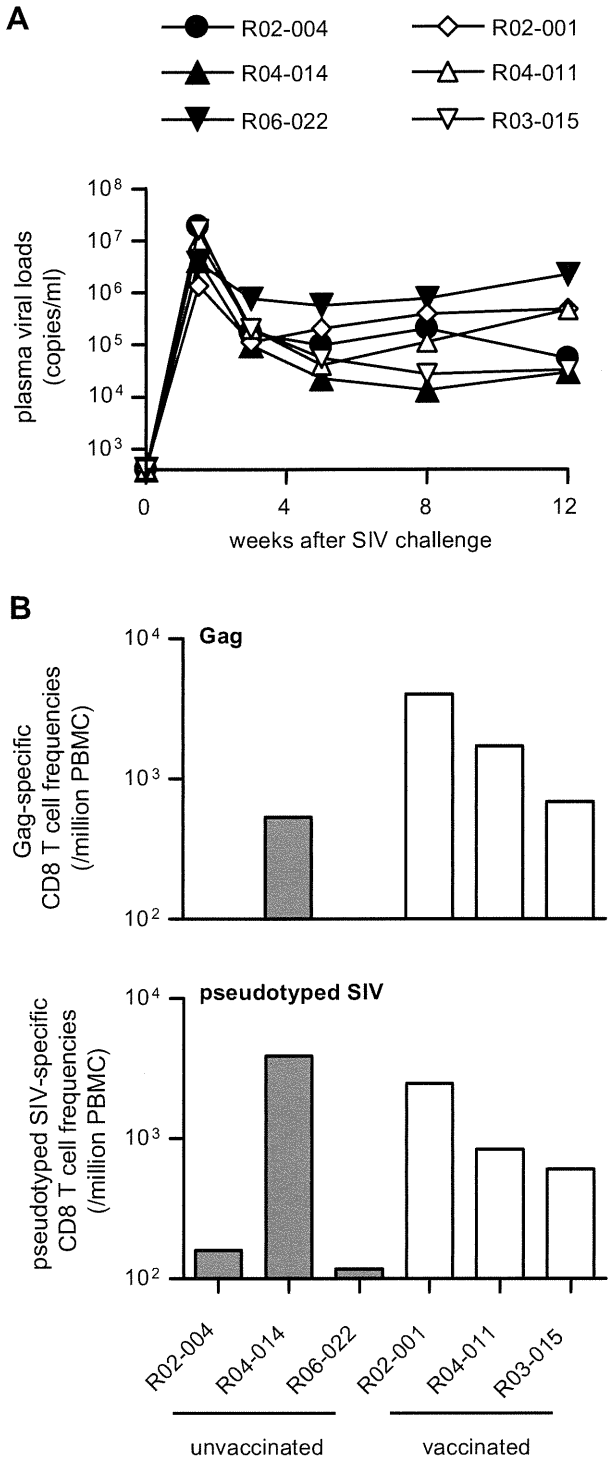

Fig. 1. CTL responses after SIVmac239 challenge in *90-088-Ij*-positive macaques. (A) Plasma viral loads after SIV challenge in unvaccinated (R02-004, R04-014, and R06-022) and DNA/SeV-Gag vaccinated animals (R02-001, R04-011, and R03-015). The viral loads (SIV gag RNA copies/ml) were determined as described previously [7]. (B) Vaccine antigen Gag-specific (upper panel) and pseudotyped SIV-specific $CD8^+$ T cell frequencies (lower panel) at week 2 after SIV challenge.

2.2. Analysis of virus-specific CTL responses

We measured virus-specific $CD8^+$ T-cell levels by flow cytometric analysis of gamma interferon (IFN-γ) induction after specific

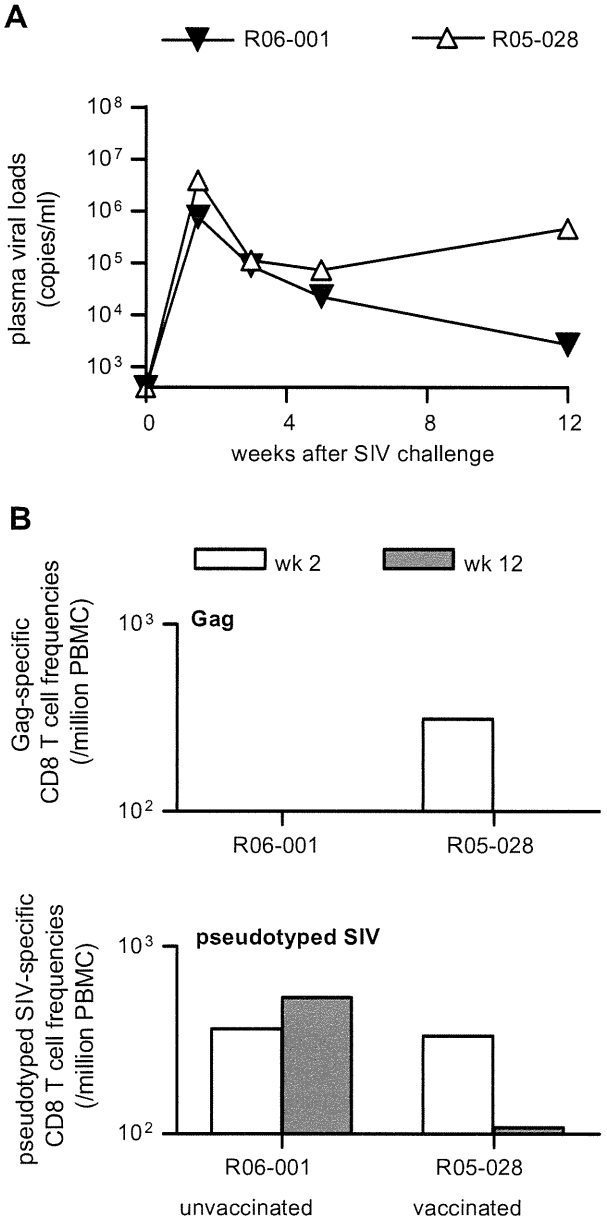

Fig. 2. CTL responses after SIVmac239 challenge in *90–120-Ib*-positive macaques. (A) Plasma viral loads after SIV challenge in unvaccinated R06–001 and SeV-Gag-vaccinated macaque R05-028. (B) Vaccine antigen Gag-specific (upper panel) and pseudotyped SIV-specific $CD8^+$ T cell frequencies (lower panel) at weeks 2 (white bars) and 12 (black bars) after SIV challenge.

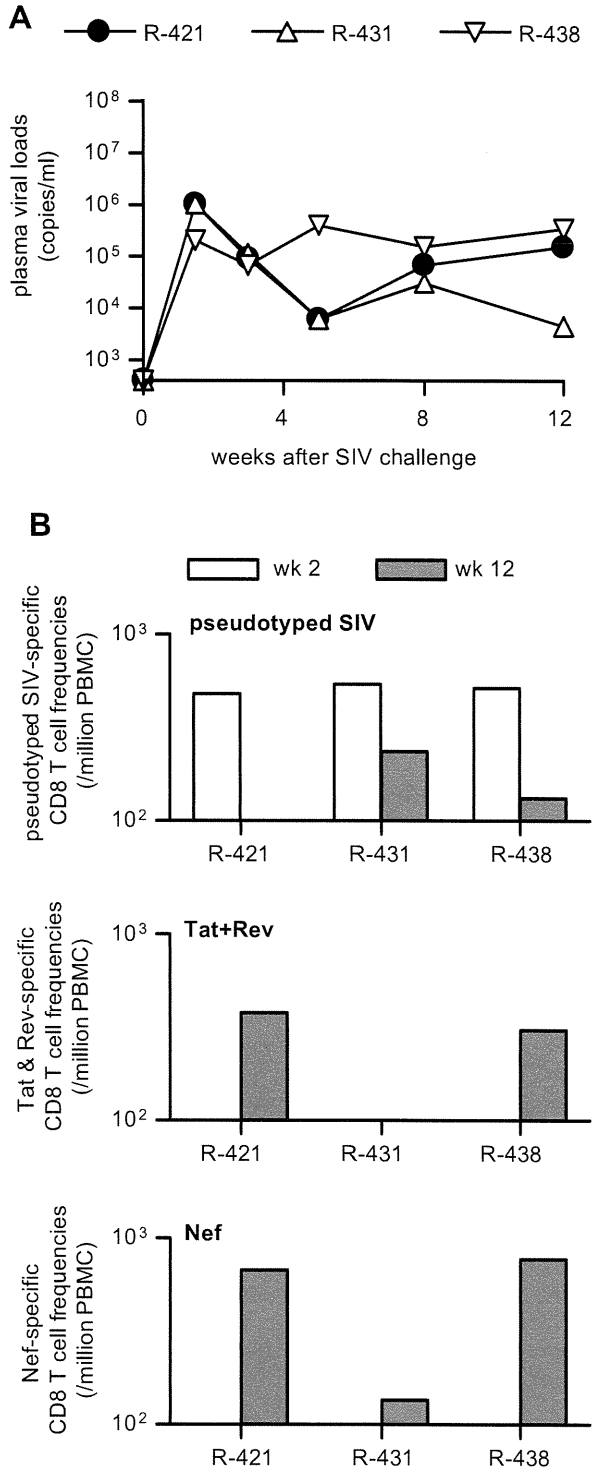

Fig. 3. CTL responses after SIVmac239 challenge in DNA-vaccinated macaques. The DNA used for the vaccination has the SIVmac239-derived region encoding Gag, Pol, Vif, and Vpx and is expected to induce pseudotyped SIV-specific CTL responses. (A) Plasma viral loads after SIV challenge in DNA vaccinated macaques R-421, R-431, and R-438. (B) Vaccine antigen (pseudotyped SIV)-specific (top panel), Tat-plus-Rev-specific (middle panel), and Nef-specific $CD8^+$ T cell frequencies (bottom panel) at weeks 2 (white bars) and 12 (black bars) after SIV challenge. In macaque R-438, CTL responses at week 5 instead of week 12 are shown.

stimulation as described previously [18,19]. Peripheral blood mononuclear cells (PBMCs) were cocultured with autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines (B-LCLs) infected with a vaccinia virus vector expressing SIVmac239 Gag for Gag-specific stimulation or a vesicular stomatitis virus G protein (VSV-G)-pseudotyped SIV for pseudotyped SIV-specific stimulation. The pseudotyped SIV was obtained by cotransfection of COS-1 cells with a VSV-G-expression plasmid and SIVGP1 DNA. Alternatively, PBMCs were cocultured with B-LCLs pulsed with peptide pools using panels of overlapping peptides spanning the entire SIVmac239 Tat, Rev, and Nef amino acid sequences. Intracellular IFN-γ staining was performed with a CytofixCytoperm kit (Becton Dickinson, Tokyo, Japan) and fluorescein isothiocyanate-conjugated anti-human CD4, peridinin chlorophyll protein-conjugated anti-human CD8, allophycocyanin-conjugated anti-human CD3, and phycoerythrin-conjugated anti-human IFN-γ monoclonal antibodies (Becton Dickinson). Specific $CD8^+$ T-cell levels were calculated by subtracting nonspecific IFN-γ^+ $CD8^+$ T-cell frequencies from those after Gag-specific, pseudotyped

	vaccine antigen					non-vaccine antigen										
	Gag				Vif	Vpr	Tat					Rev		Nef		
	165	333	375	376	143	73	23	115	120	122	125	45	50	63	100	124
wk 5																
R-421					++											
R-431					+											
R-438	++		+							++						
wk 12																
R-421		++			++				+		+	+	+			++
R-431					+		+			++						
R-438	++			++		+		++						++	++	

Fig. 4. Viral mutations in DNA-vaccinated macaques. Plasma viral genome sequencing was performed as described previously [18] to determine mutations resulting in amino acid substitutions in SIV Gag, Pol, Vif, Vpx, Vpr, Tat, Rev, and Nef antigens (except for Env) at weeks 5 and 12 in DNA-vaccinated macaques. The amino acid positions showing mutant sequences dominantly (++) or equivalently with wild type (+) are shown. While we found a mutation leading to a lysine-to-arginine alteration at the 40th amino acid in Rev in all animals, this mutation is not shown because the wild-type sequence at this position in the SIVmac239 molecular clone is considered to be a suboptimal nucleotide that frequently reverts to an alternative sequence in vivo [18,23].

SIV-specific, or peptide-specific stimulation. Specific $CD8^+$ T-cell levels lower than 100 per million PBMCs were considered negative.

3. Results and discussion

In our previous SIVmac239 challenge experiments, the prophylactic DNA/SeV-Gag vaccination did not result in viral control in rhesus macaques possessing the MHC-I haplotype *90-088-Ij*. These vaccinated animals showed similar levels of plasma viral loads as those in unvaccinated *90-088-Ij*-positive animals after SIV challenge (Fig. 1A). Analysis of virus-specific $CD8^+$ T-cell responses using PBMCs at week 2 after challenge showed equivalent Gag-specific and pseudotyped SIV-specific (Gag-, Pol-, Vif-, and Vpx-specific) CTL responses in all three vaccinees (Fig. 1B). Pseudotyped SIV-specific CTL responses were also detected in all three unvaccinated animals, but Gag-specific CTL responses were undetectable in two out of the three; even the Gag-specific CTL responses detected in macaque R04-014 were much lower than pseudotyped SIV-specific CTL responses, indicating dominant induction of CTL responses specific for SIV antigens other than Gag (Fig. 1B). Thus, in the acute phase of SIV infection, SIV non-Gag antigen-specific CTL responses were dominantly induced in unvaccinated *90-088-Ij*-positive macaques, whereas vaccine antigen (Gag)-specific CTL responses were dominant in *90-088-Ij*-positive vaccinees.

We then analyzed another vaccinees that failed to control a SIVmac239 challenge; these macaques were vaccinated with SeV-Gag alone or DNA alone. First, we compared post-challenge CTL responses in unvaccinated and SeV-Gag-vaccinated macaques possessing the MHC-I haplotype *90-120-Ib*. Both macaques failed to control SIV replication after challenge (Fig. 2A). In the unvaccinated animal R06-001, Gag-specific CTL responses were undetectable but pseudotyped SIV-specific CTL responses were induced efficiently at weeks 2 and 12 (Fig. 2B). In contrast, Gag-specific CTL responses were induced efficiently at week 2 in the SeV-Gag-vaccinated animal R05-028 (Fig. 2B). At week 12, Gag-specific CTL responses became undetectable while pseudotyped SIV-specific CTL responses were still detectable in this animal. These results indicate that, in the acute phase after SIVmac239 challenge, the unvaccinated *90-120-Ib*-positive macaque dominantly elicited SIV non-Gag antigen-specific CTL responses whereas the SeV-Gag-vaccinated *90-120-Ib*-positive macaque dominantly induced vaccine antigen (Gag)-specific CTL responses.

Next, we analyzed post-challenge CTL responses in three DNA-vaccinated macaques. These animals failed to control SIVmac239 replication after challenge (Fig. 3A). The DNA used for the vaccination and the pseudotyped SIV genome both have the same SIVmac239-derived region encoding Gag, Pol, Vif, and Vpx, thus expected to induce pseudotyped SIV-specific CTL responses. Pseudotyped SIV-specific CTL responses, namely vaccine antigen-specific CTL responses, were induced efficiently at week 2 but diminished after that in all three animals (Fig. 3B). In contrast, Tat/Rev- and Nef-specific CTL responses were undetectable at week 2 but induced later (Fig. 3B). Again, vaccine antigen-specific CTL responses were dominantly induced in the acute phase after SIV challenge and non-vaccine antigen-specific CTL responses were elicited later.

All three animals showed viral genome mutations leading to amino acid substitutions in Gag or Vif at week 5 (Fig. 4). Further analysis indicated that viral mutations in vaccine antigen-coding regions appeared earlier than those in other regions. These results may reflect selective pressure on SIV by vaccine antigen-specific CTL responses dominantly induced in the acute phase, although it remains undetermined whether these mutations are CTL escape ones. Disappearance of vaccine antigen-specific CTL responses at week 12 may be explained by rapid selection of CTL escape mutations in vaccine antigen-coding regions. However, analysis using peptides found Gag-specific CTL responses in macaques R-421 and R-431 that had no gag mutations at week 5 (data not shown), suggesting involvement of immunodominance [20] in the disappearance of vaccine antigen-specific CTL responses at week 12.

In summary, the present study indicates that vaccine antigen-specific CTL responses are induced dominantly in the acute phase after viral exposure, with delayed induction of CTL responses specific for SIV non-vaccine antigens (SIV antigens other than vaccine antigens). While this delay previously-observed in vaccine-based SIV controllers [10] can be explained not only by immunodominance but also by reduction in viral loads, the delay in vaccinated non-controllers in the present study might reflect the immunodominance in CTL responses. Thus, in development of a prophylactic, CTL-inducing AIDS vaccine, it is important to select vaccine antigens leading to effective CTL responses post-viral

exposure [21,22]. These results imply a significant influence of prophylactic vaccination on the immunodominance pattern of CTL responses post-viral exposure, providing insights into antigen design in development of a CTL-inducing AIDS vaccine.

Acknowledgments

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Impact of Vaccination on Cytotoxic T Lymphocyte Immunodominance and Cooperation against Simian Immunodeficiency Virus Replication in Rhesus Macaques

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Cytotoxic T lymphocyte (CTL) responses play a central role in viral suppression in human immunodeficiency virus (HIV) infections. Prophylactic vaccination resulting in effective CTL responses after viral exposure would contribute to HIV control. It is important to know how CTL memory induction by vaccination affects postexposure CTL responses. We previously showed vaccine-based control of a simian immunodeficiency virus (SIV) challenge in a group of Burmese rhesus macaques sharing a major histocompatibility complex class I haplotype. $Gag_{206-216}$ and $Gag_{241-249}$ epitope-specific CTL responses were responsible for this control. In the present study, we show the impact of individual epitope-specific CTL induction by prophylactic vaccination on postexposure CTL responses. In the acute phase after SIV challenge, dominant $Gag_{206-216}$-specific CTL responses with delayed, naive-derived $Gag_{241-249}$-specific CTL induction were observed in $Gag_{206-216}$ epitope-vaccinated animals with prophylactic induction of single $Gag_{206-216}$ epitope-specific CTL memory, and vice versa in $Gag_{241-249}$ epitope-vaccinated animals with single $Gag_{241-249}$ epitope-specific CTL induction. Animals with $Gag_{206-216}$-specific CTL induction by vaccination selected for a $Gag_{206-216}$-specific CTL escape mutation by week 5 and showed significantly less decline of plasma viral loads from week 3 to week 5 than in $Gag_{241-249}$ epitope-vaccinated animals without escape mutations. Our results present evidence indicating significant influence of prophylactic vaccination on postexposure CTL immunodominance and cooperation of vaccine antigen-specific and non-vaccine antigen-specific CTL responses, which affects virus control. These findings provide great insights into antigen design for CTL-inducing AIDS vaccines.

Human immunodeficiency virus (HIV) infection induces chronic, persistent viral replication leading to AIDS onset in humans. Virus-specific cytotoxic T lymphocyte (CTL) responses play a central role in the resolution of acute peak viremia (3, 4, 13, 22, 28) but mostly fail to contain viral replication in the natural course of HIV infection. Vaccination resulting in more effective CTL responses after viral exposure than in natural HIV infections would contribute to HIV control (30, 33). CTL memory induction by prophylactic vaccination may lead to efficient secondary CTL responses, but naive-derived primary CTL responses specific for viral nonvaccine antigens can also be induced after viral exposure. It is important to know how CTL memory induction by vaccination affects these postexposure CTL responses.

Cumulative studies on HIV-infected individuals have shown association of HLA genotypes with rapid or delayed AIDS progression (5, 14, 31, 34). For instance, most of the HIV-infected individuals possessing *HLA-B*57* have been indicated to show a better prognosis with lower viral loads, implicating HLA-B*57-restricted epitope-specific CTL responses in this viral control (1, 8, 23, 24). Indian rhesus macaques possessing certain major histocompatibility complex class I (MHC-I) alleles, such as *Mamu-A*01*, *Mamu-B*08*, and *Mamu-B*17*, tend to show simian immunodeficiency virus (SIV) control (19, 25, 36). This implies possible HIV control by induction of particular effective CTL responses (2, 7, 12, 16, 27).

Recent trials of prophylactic T-cell-based vaccines in macaque AIDS models have indicated the possibility of reduction in post-challenge viral loads (6, 15, 17, 21, 35). We previously developed a prophylactic AIDS vaccine consisting of a DNA prime and a boost with a Sendai virus (SeV) vector expressing SIVmac239 Gag (SeV-Gag) (20). Our trial showed vaccine-based control of an SIVmac239 challenge in a group of Burmese rhesus macaques sharing the MHC-I haplotype *90-120-Ia* (21). Animals possessing *90-120-Ia* dominantly elicited Mamu-A1*043:01 (GenBank accession number AB444869)-restricted $Gag_{206-216}$ (IINEEAADWDL) epitope-specific and Mamu-A1*065:01 (AB444921)-restricted $Gag_{241-249}$ (SSVDEQIQW) epitope-specific CTL responses after SIV challenge and selected for viral *gag* mutations, GagL216S (leading to a leucine [L]-to-serine [S] substitution at amino acid [aa] 216 in Gag) and GagD244E (aspartic acid [D]-to-glutamic acid [E] at aa 244), resulting in escape from CTL recognition with viral fitness costs in the chronic phase (9, 26). Vaccinees possessing *90-120-Ia* failed to control a challenge with a mutant SIV carrying these two CTL escape mutations, indicating that $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses play a crucial role in the vaccine-based control of wild-type SIVmac239 replication

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TABLE 1 Animals analyzed in this study

Group	No. of animals	Vaccination[a]	SIV-specific CTL response postboost
I	6	None	None
II	5	Gag (pCMV-SHIVdEN DNA prime, SeV-Gag boost)	Gag-specific CTL
III	6	$Gag_{241-249}$-specific (p$Gag_{236-250}$-EGFP-N1 DNA prime, SeV-$Gag_{236-250}$-EGFP boost)	$Gag_{241-249}$-specific CTL
IV	5	$Gag_{206-216}$-specific (p$Gag_{202-216}$-EGFP-N1 DNA prime, SeV-$Gag_{202-216}$-EGFP boost)	$Gag_{206-216}$-specific CTL

[a] All animals were challenged with SIVmac239.

(10). Furthermore, in an SIVmac239 challenge experiment with *90-120-Ia*-positive rhesus macaques that received a prophylactic vaccine expressing the $Gag_{241-249}$ epitope fused with enhanced green fluorescent protein (EGFP), this single-epitope vaccination resulted in control of SIVmac239 replication with dominant induction of $Gag_{241-249}$-specific CTL responses in the acute phase postchallenge (32).

Thus, it is hypothesized that induction of single $Gag_{206-216}$ or $Gag_{241-249}$ epitope-specific CTL responses by vaccination may result in different patterns of CTL immunodominance and viral replication after SIV challenge. In the present study, we analyzed the impact of prophylactic vaccination inducing single $Gag_{206-216}$ epitope-specific CTL responses on SIV control in *90-120-Ia*-positive macaques and compared the results with those of vaccination inducing single $Gag_{241-249}$ epitope-specific CTL responses. This analysis revealed differences in CTL responses and patterns of viral control after SIV challenge between these vaccinated groups, indicating significant effects of prophylactic vaccination on postexposure CTL immunodominance and cooperation of vaccine antigen-specific and non-vaccine antigen-specific CTL responses.

MATERIALS AND METHODS

Animal experiments. Animal experiments were conducted through the Cooperative Research Program at Tsukuba Primate Research Center, National Institute of Biomedical Innovation, with the help of the Corporation for Production and Research of Laboratory Primates. Blood collection, vaccination, and virus challenge were performed under ketamine anesthesia. All animals were maintained in accordance with the Guideline for Laboratory Animals of the National Institute of Infectious Diseases.

Five Burmese rhesus macaques (*Macaca mulatta*) possessing the MHC-I haplotype *90-120-Ia* (26) (group IV) received a DNA-prime/SeV-boost vaccine eliciting $Gag_{206-216}$-specific CTL responses followed by an SIVmac239 challenge and were compared with three groups (I, II, and III) of *90-120-Ia*-positive animals reported previously (10, 32) (Table 1). Group I animals ($n = 6$) received no vaccination, while group II animals ($n = 5$) received a DNA-prime/SeV-boost vaccine eliciting Gag-specific CTL responses. The DNA, CMV-SHIVdEN, used for the vaccination was constructed from a simian/human immunodeficiency virus ($SHIV_{MD14YE}$) molecular clone DNA with *env* and *nef* deleted (29) and has the genes encoding SIVmac239 Gag, Pol, Vif, and Vpx; SIVmac239–HIV-1 chimeric Vpr; and HIV-1 Tat and Rev (21). In group II animals, CTL responses were undetectable after DNA prime but Gag-specific CTL responses became detectable after SeV-Gag boost. Group III animals ($n = 6$) received a DNA-prime/SeV-boost vaccine eliciting $Gag_{241-249}$-specific CTL responses. A p$Gag_{236-250}$-EGFP-N1 DNA and an SeV-$Gag_{236-250}$-EGFP vector, both expressing an SIVmac239 $Gag_{236-250}$ (IAGTTSSVDEQ IQWM)-EGFP fusion protein, were used for the group III vaccination. After the SeV-$Gag_{236-250}$-EGFP boost, group III animals induced $Gag_{241-249}$-specific CTL responses; the animals showed no $Gag_{236-250}$-specific $CD4^+$ T-cell responses but elicited SeV/EGFP-specific $CD4^+$ T-cell responses (32). For the group IV vaccination, A p$Gag_{202-216}$-EGFP-N1 DNA and an SeV-$Gag_{202-216}$-EGFP vector, both expressing an SIVmac239 $Gag_{202-216}$ (IIRDIINEEAADWDL)-EGFP fusion protein, were used (Fig. 1). Approximately 3 months after the boost, all animals were challenged intravenously with 1,000 50% tissue culture infective doses of SIVmac239 (11). In our previous study (32), the unvaccinated and the control-vaccinated

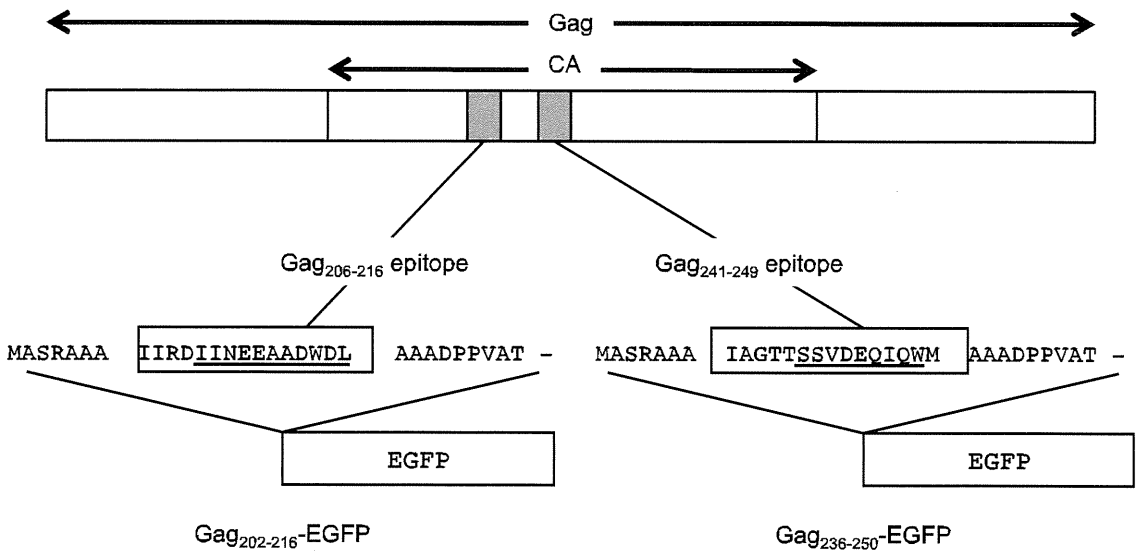

FIG 1 Schema of the cDNA constructs encoding $Gag_{202-216}$-EGFP and $Gag_{236-250}$-EGFP fusion proteins. A DNA fragment that encodes a 31-mer peptide (boxes) including the $Gag_{202-216}$ or $Gag_{236-250}$ sequence (underlining) was introduced into the 5′ end of the EGFP cDNA.

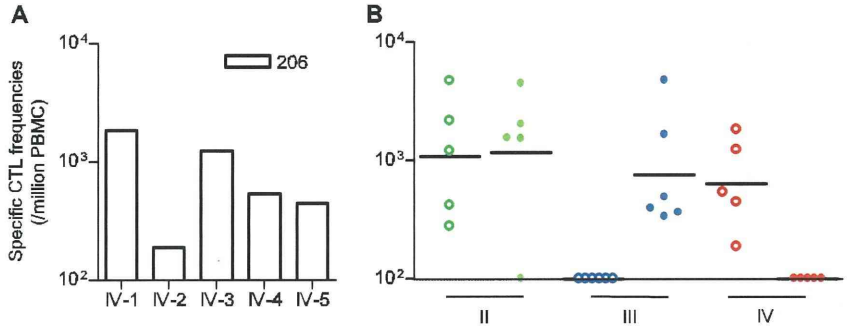

FIG 2 $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses after prophylactic vaccination. (A) $Gag_{206-216}$-specific $CD8^+$ T-cell frequencies 1 week after SeV-$Gag_{202-216}$–EGFP boost in group IV macaques (open boxes). (B) $Gag_{206-216}$-specific (open circles) and $Gag_{241-249}$-specific (closed circles) $CD8^+$ T-cell frequencies 1 week after boost in group II (green), III (blue), and IV (red) macaques. The bars indicate the geometric mean of each group. No animal showed detectable Gag-specific CTL responses before the boost.

animals receiving a DNA and an SeV expressing EGFP showed no significant differences in viral loads after SIV challenge.

Analysis of antigen-specific CTL 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 (21). Peripheral blood mononuclear cells (PBMCs) were cocultured with autologous herpesvirus papioimmortalized B-lymphoblastoid cell lines pulsed with 1 μM SIVmac239 $Gag_{206-216}$ (IINEEAADWDL), $Gag_{241-249}$ (SSVDEQIQW), or $Gag_{367-381}$ (ALKEALAPVPIPFAA) peptide for $Gag_{206-216}$-specific, $Gag_{241-249}$-specific, or $Gag_{367-381}$-specific stimulation. Intracellular IFN-γ staining was performed with a CytofixCytoperm kit (BD, Tokyo, Japan) and fluorescein isothiocyanate-conjugated anti-human CD4 (BD), peridinin chlorophyll protein-conjugated anti-human CD8 (BD), allophycocyanin (APC)-Cy7-conjugated anti-human CD3 (BD), and phycoerythrin (PE)-conjugated anti-human IFN-γ (Biolegend, San Diego, CA) monoclonal antibodies. Specific T-cell levels were calculated by subtracting nonspecific IFN-γ T-cell frequencies from those after peptide-specific stimulation. Specific T-cell levels lower than 100 per million PBMCs were considered negative.

Sequencing of the viral genome. Plasma RNA was extracted using the High Pure viral RNA kit (Roche Diagnostics, Tokyo, Japan). Fragments corresponding to nucleotides from 1231 to 2958 (containing the entire *gag* region) in the SIVmac239 genome (GenBank accession number M33262) were amplified by nested reverse transcription (RT)-PCR. The PCR products were sequenced using dye terminator chemistry and an automated DNA sequencer (Applied Biosystems, Tokyo, Japan).

Statistical analysis. Statistical analyses were performed using R software (R Development Core Team). Differences in geometric means of plasma viral loads were examined by one-way analysis of variance (ANOVA) and Tukey-Kramer's multiple-comparison test. Plasma viral loads at week 3 were examined for differences between group III and groups II and IV by analysis of covariance (ANCOVA) with week 5 viral loads as a covariate.

RESULTS

CTL responses after prophylactic vaccination. We previously reported the efficacy of vaccination eliciting whole Gag-specific or single $Gag_{241-249}$ epitope-specific CTL memory against SIVmac239 challenge (10, 32). In the present study, we examined the efficacy of prophylactic induction of single $Gag_{206-216}$ epitope-specific CTL memory against SIVmac239 challenge and compared the results with those of the previous experiments.

Five Burmese rhesus macaques possessing MHC-I haplotype *90-120-Ia* received a DNA-prime/SeV-boost vaccine eliciting single $Gag_{206-216}$ epitope-specific CTL responses. A plasmid DNA (p$Gag_{202-216}$-EGFP-N1) and an SeV (SeV-$Gag_{202-216}$-EGFP) vector, both expressing an SIVmac239 $Gag_{202-216}$-EGFP fusion pro-

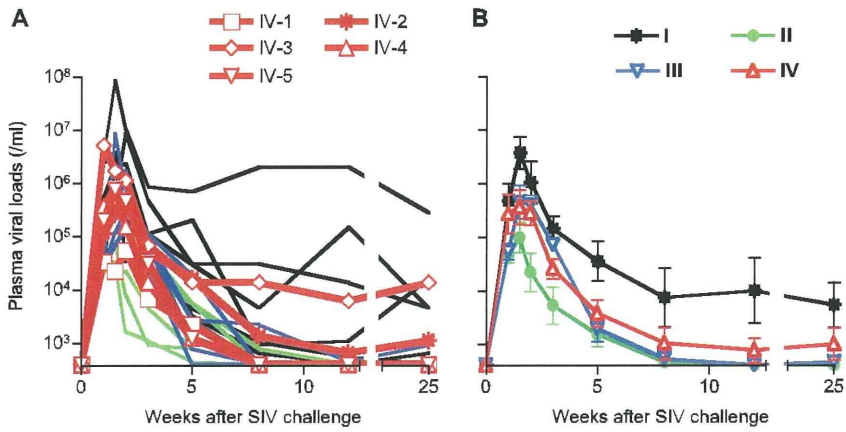

FIG 3 Plasma viral loads after SIVmac239 challenge. The plasma viral loads in group I, group II, group III, and group IV animals were determined as described previously (21). The lower limit of detection was approximately 4×10^2 copies/ml. (A) Changes in plasma viral loads (SIV *gag* RNA copies/ml plasma) after challenge. (B) Changes in geometric means of plasma viral loads after challenge. Groups II and III (but not group IV) showed significantly lower set point viral loads than group I ($P = 0.0390$ between groups I and II, $P = 0.0404$ between groups I and III, and $P > 0.05$ between groups I and IV at week 25 by one-way ANOVA and Tukey-Kramer's multiple-comparison test).

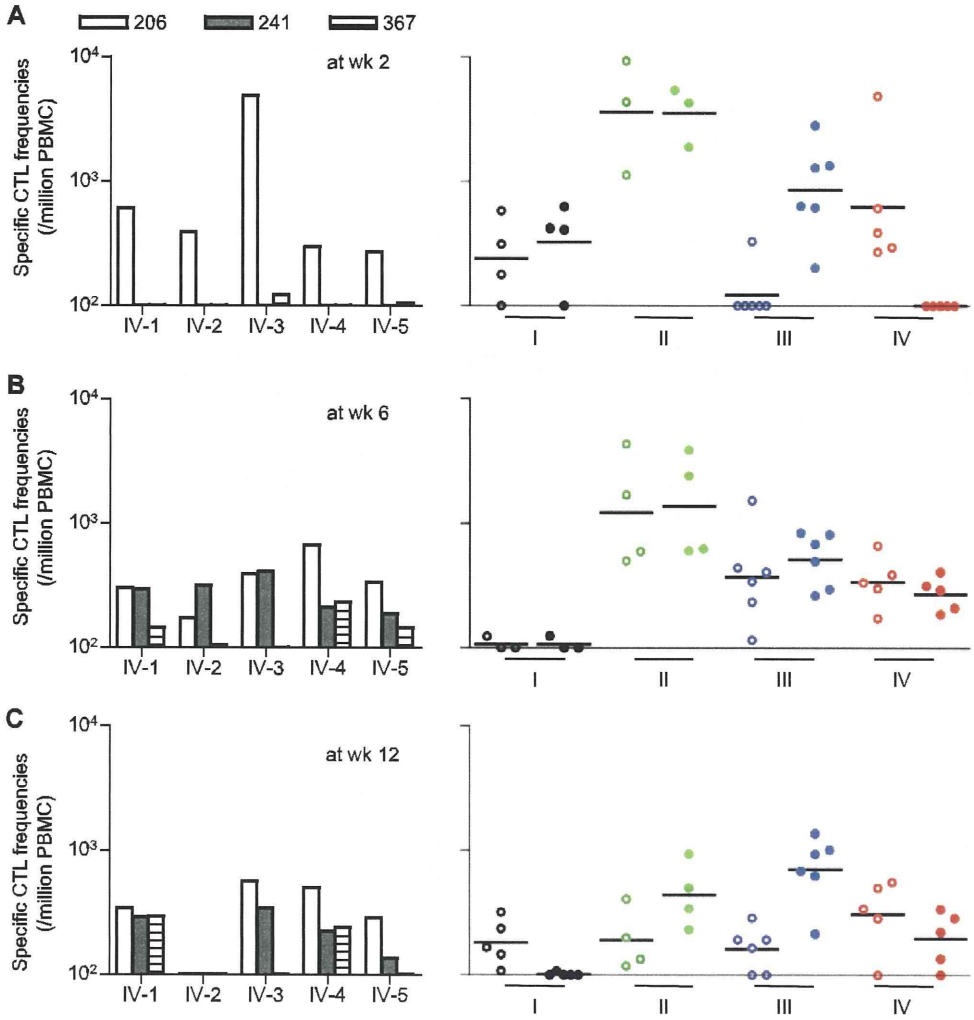

FIG 4 $Gag_{206\text{-}216}$-specific and $Gag_{241\text{-}249}$-specific CTL responses after SIVmac239 challenge. CTL responses at week 2 (A), week 6 (B), and week 12 (C) are shown. In the graphs on the left, $Gag_{206\text{-}216}$-specific (open boxes), $Gag_{241\text{-}249}$-specific (closed boxes), and $Gag_{367\text{-}381}$-specific (striped boxes) $CD8^+$ T-cell frequencies in group IV macaques are shown. On the right, $Gag_{206\text{-}216}$-specific (open circles) and $Gag_{241\text{-}249}$-specific (closed circles) $CD8^+$ T-cell frequencies in group I (black), II (green), III (blue), and IV (red) macaques are shown. The bars indicate the geometric mean of each group. Samples from macaques I-1, I-6, II-1, and II-3 at week 2; macaques I-1, I-2, I-6, and II-5 at week 6; and macaques I-1 and II-5 at week 12 were unavailable for this analysis. Statistical analyses among four groups at week 12 revealed significant differences in $Gag_{241\text{-}249}$-specific CTL levels (I and III, $P < 0.0001$; I and II, and III and IV, $P < 0.01$; I and IV, II and III, and II and IV, $P > 0.05$ by one-way ANOVA and Tukey-Kramer's multiple-comparison test) but not in $Gag_{206\text{-}216}$-specific CTL levels ($P > 0.05$ by one-way ANOVA).

tein, were used for the vaccination (Fig. 1). We confirmed $Gag_{206\text{-}216}$-specific CTL responses 1 week after SeV-$Gag_{202\text{-}216}$-EGFP boost in all five animals (Fig. 2A). As expected, no $Gag_{241\text{-}249}$-specific CTL responses were detected in these animals. No $Gag_{202\text{-}216}$-specific $CD4^+$ T-cell responses were detected in the animals except for one (IV-5) showing marginal levels of responses (data not shown).

Plasma viral loads after SIV challenge. We compared these five animals (referred to as group IV) with other groups (I, II, and III) of *90-120-Ia*-positive macaques reported previously (Table 1). Group I animals ($n = 6$) received no vaccination, group II ($n = 5$) received a DNA-prime/SeV-boost vaccine eliciting whole Gag-specific CTL responses, and group III ($n = 6$) received a DNA-prime/SeV-boost vaccine eliciting single $Gag_{241\text{-}249}$ epitope-specific CTL responses. Both $Gag_{206\text{-}216}$-specific and $Gag_{241\text{-}249}$-specific CTL responses were detectable after SeV-Gag boost in four of five group II animals except for one animal (II-3), in which $Gag_{206\text{-}216}$-specific, but not $Gag_{241\text{-}249}$-specific, CTL responses were detected. In all group III animals, $Gag_{241\text{-}249}$-specific CTL responses were confirmed, while no $Gag_{206\text{-}216}$-specific CTL responses were detected after SeV-$Gag_{236\text{-}250}$-EGFP boost (Fig. 2B).

After SIVmac239 challenge, all animals were infected and showed plasma viremia during the acute phase. Plasma viremia was maintained in five of six unvaccinated animals in group I but became undetectable in one animal (I-2) at week 12. In contrast, all animals in groups II and III contained SIV replication with significantly reduced plasma viral loads compared to group I at the set point. In group IV, however, vaccine efficacy was not so clear; while three out of five animals contained SIV replication, the remaining two (IV-2 and IV-3) failed to control viral replication with persistent plasma viremia (Fig. 3).

Gag-specific CTL responses after SIV challenge. We then measured $Gag_{206\text{-}216}$-specific and $Gag_{241\text{-}249}$-specific CTL responses after SIVmac239 challenge by detection of peptide-

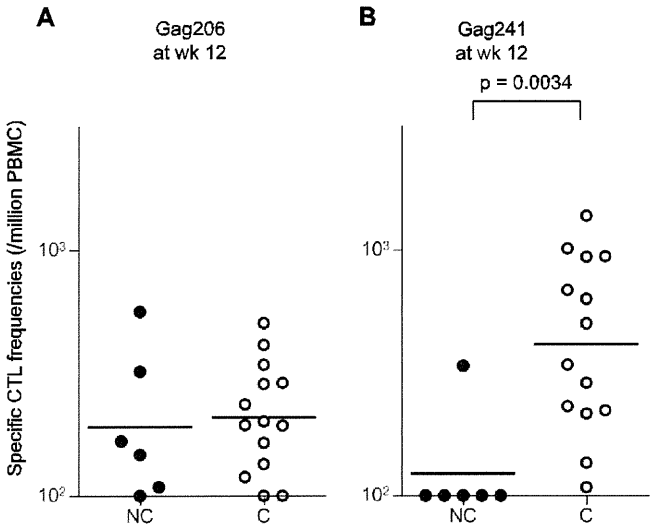

FIG 5 Comparison of $Gag_{206-216}$-specific or $Gag_{241-249}$-specific CTL responses in noncontrollers and controllers at week 12. (A) $Gag_{206-216}$-specific $CD8^+$ T-cell frequencies in noncontrollers (NC; closed circles) and controllers (C; open circles). (B) $Gag_{241-249}$-specific $CD8^+$ T-cell frequencies in noncontrollers and controllers. $Gag_{241-249}$-specific CTL levels in controllers were significantly higher than those in noncontrollers ($P = 0.0034$ by Mann-Whitney test). The bars indicate the geometric mean of each group. Data on a noncontroller (I-1) and a controller (II-5) were unavailable.

specific IFN-γ induction. At week 2 (Fig. 4A), most animals in groups I and II elicited both $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses, whereas group III animals induced $Gag_{241-249}$-specific CTL responses dominantly. Remarkably, all animals in group IV showed efficient $Gag_{206-216}$-specific CTL responses without detectable $Gag_{241-249}$-specific CTL responses at week 2. These results indicate dominant $Gag_{206-216}$-specific CTL responses with delayed induction of $Gag_{241-249}$-specific CTL responses postchallenge in group IV animals with prophylactic $Gag_{206-216}$-specific CTL induction, and vice versa in group III animals.

At week 6 (Fig. 4B), efficient $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses were observed in all vaccinated animals in groups II, III, and IV, but not in group I. $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses were induced equivalently even in groups III and IV. We also examined subdominant $Gag_{367-381}$ epitope-specific CTL responses, which were undetectable at week 2 but became detectable at week 6 in most group IV animals (Fig. 4, graphs on left). At week 12 (Fig. 4C), however, different CTL immunodominance patterns were observed among the groups. $Gag_{241-249}$-specific CTL levels were higher than $Gag_{206-216}$-specific levels in groups II and III but were reduced in groups I and IV. Interestingly, comparison between the animals with persistent viremia (referred to as noncontrollers) and those controlling SIV replication (referred to as controllers) revealed significant differences in $Gag_{241-249}$-specific CTL levels, but not in $Gag_{206-216}$-specific levels, at week 12 ($P = 0.0034$ by Mann-Whitney test) (Fig. 5).

Selection of a CTL escape mutation. Next, we examined viral genome *gag* sequences at weeks 5 and 12 after challenge to determine whether CTL escape mutations were selected in these animals (Table 2). At week 5, a mutation leading to an L-to-S substitution at the 216th residue in Gag (L216S) was selected in all the group II animals. This GagL216S change results in escape from $Gag_{206-216}$-specific CTL recognition, as described previously (21). All the group IV animals with $Gag_{206-216}$-specific CTL induction also showed rapid selection of this CTL escape mutation at week 5. Analysis at week 3 found the GagL216S mutation dominant in two (II-2 and II-5) group II and two (IV-1 and IV-3) group IV animals (data not shown). However, animals in group III showed no *gag* mutations at week 5, except for one animal (III-5) selecting a mutation leading to an L-to-F substitution at the 216th residue. Later, at week 12, the $Gag_{206-216}$-specific CTL escape mutation, GagL216S, was selected even in group III animals. No animals showed mutations around the $Gag_{241-249}$ epitope-coding region even at week 12. These results indicate that selection of this $Gag_{206-216}$-specific CTL escape mutation may be accelerated by prophylactic vaccination inducing $Gag_{206-216}$-specific CTL responses. On the other hand, in group III animals with single $Gag_{241-249}$ epitope-specific CTL induction, selection of a $Gag_{206-216}$-specific CTL escape mutation was delayed but was observed before selection of a $Gag_{241-249}$-specific CTL escape mutation, suggesting strong selective pressure by delayed $Gag_{206-216}$-specific CTL responses after SIV challenge.

In order to see the effect of rapid selection of the $Gag_{206-216}$-specific CTL escape mutation on SIV control, we compared plasma viral loads at weeks 3 and 5 between groups II and IV (referred to as group II+IV) with rapid selection of the GagL216S

TABLE 2 Selection of a CTL escape mutation

Group	Macaque ID	Amino acid change for Gag residues[b]: 206–216 Wk 5	206–216 Wk 12	241–249 Wk 5	241–249 Wk 12
I	I-1	None	ND	None	ND
	I-2[a]	None	L216S	None	None
	I-3	None	L216S	None	None
	I-4	None	None	None	None
	I-5	None	None	None	None
	I-6	None	None	None	None
II	II-1[a]	L216S	ND	None	ND
	II-2[a]	L216S	ND	None	ND
	II-3[a]	L216S	ND	None	ND
	II-4[a]	L216S	ND	None	ND
	II-5[a]	L216S	ND	None	ND
III	III-1[a]	None	L216S	None	None
	III-2[a]	None	L216S	None	None
	III-3[a]	None	NA	None	NA
	III-4[a]	None	NA	None	NA
	III-5[a]	L216F	L216S	None	None
	III-6[a]	None	L216S	None	None
IV	IV-1[a]	L216S	L216S	None	None
	IV-2	L216S	L216S	None	None
	IV-3	L216S	L216S	None	None
	IV-4[a]	L216S	L216S	None	None
	IV-5[a]	L216S	NA	None	NA

[a] Animals that controlled SIV replication at week 12 (controllers).

[b] Plasma viral gag genome mutations were examined at weeks 5 and 12. Amino acid substitutions in $Gag_{206-216}$ and $Gag_{241-249}$ epitope regions are shown. L216S results in viral escape from $Gag_{206-216}$-specific CTL recognition. It remains undetermined whether L216F results in CTL escape. ND, not determined; NA, not determined because Gag fragments were unable to be amplified from plasma RNA.

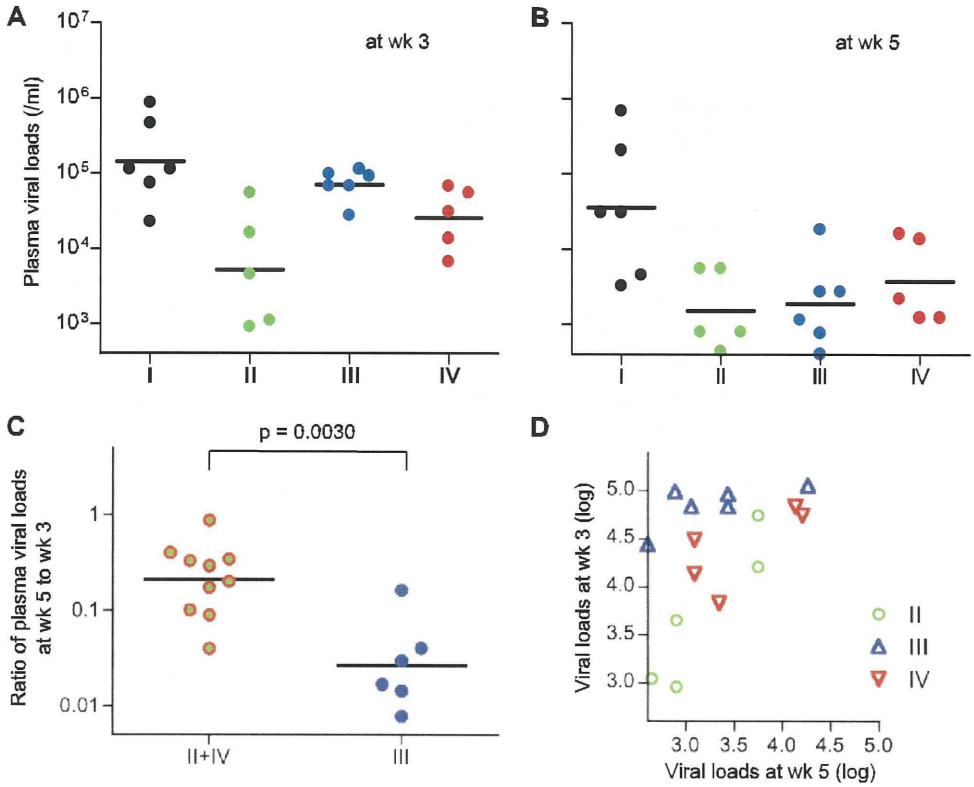

FIG 6 Comparison of plasma viral loads at weeks 3 and 5 among four groups. (A) Plasma viral loads at week 3 in group I, II, III, and IV animals. (B) Plasma viral loads at week 5 in group I, II, III, and IV animals. (C) Comparison of ratios of plasma viral loads at week 5 to week 3 in group II+IV animals and group III animals. The ratios in group III were significantly lower than those in group II+IV ($P = 0.0030$ by Mann-Whitney test). The bars indicate the geometric mean of each group. (D) Scatter plots between plasma viral loads at weeks 3 and 5 in group II, III, and IV animals.

mutation and group III without the mutation at week 5 (Fig. 6). Ratios of plasma viral loads at week 5 to week 3 in group III were significantly lower than those in group II+IV ($P = 0.0030$ by Mann-Whitney test) (Fig. 6C). To confirm this result, we examined the difference in week 3 viral loads between groups III and II+IV by ANCOVA, with week 5 viral loads as a covariate. This analysis revealed that week 3 viral loads controlled for by week 5 viral loads were significantly higher in group III than those in group II+IV (Fig. 6D and Table 3); i.e., the decline in viral loads from week 3 to week 5 was significantly sharper in group III than in group II+IV, possibly reflecting viral escape from suppressive pressure by $Gag_{206-216}$-specific CTL responses in the latter group during this period (from week 3 to week 5).

DISCUSSION

In the present study, we analyzed the impact of vaccination inducing single $Gag_{206-216}$ epitope-specific CTL memory on postchallenge CTL responses and SIV control in *90-120-Ia*-positive macaques and then compared the results with those of vaccination inducing single $Gag_{241-249}$ epitope-specific CTL responses. Our results indicate that these prophylactic vaccinations result in different patterns of $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL immunodominance and cooperation after SIVmac239 challenge.

Unvaccinated *90-120-Ia*-positive macaques (group I) showed both $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL responses after SIV challenge. In group IV animals with prophylactic induc-

TABLE 3 ANCOVA on week 3 viral loads with week 5 viral loads as a covariate between groups III and II+IV

ANOVA	Parameter	SS[a]	df[b]	MS[c]	*F*	*P* value
Homogeneity of slopes of regression	Group × slope	0.304	1	0.304	2.099	0.173
	Residual	1.735	12	0.145		
	Total	2.038	13	0.157		
Difference in week 3 viral loads with week 5 viral loads as a covariate between groups III and II+IV	Effect and group	1.106	1	1.106	7.052	0.020
	Residual	2.038	13	0.157		
	Total	3.144	14	0.225		

[a] SS, sum of squares.
[b] df, degrees of freedom.
[c] MS, mean squares.