

デルと+7のgp120分子モデルを比較した。シミュレーション時間5ns~15nsの構造を重ね合わせると、V3の先端部はV3配列の荷電量が+3では β 20- β 21ループとは反対方向に向き、荷電量が+7では β 20- β 21ループの方向に向いていた。したがって、gp120コアが同じアミノ酸配列であっても、V3配列の荷電量によってV3の配置が異なることが示された。

次に、CD4結合ループへのV3配列の荷電量の影響を調べるため、V3配列の荷電量が+3のgp120分子モデルと+7のgp120分子モデルの15nsにおける構造を重ね合わせると、荷電量が+7であるgp120のCD4結合ループは+3であるgp120よりもV4ループの方向に位置している。(図1)これは荷電量が+7のgp120は+3のp120よりもCD4結合部位が広いことを示唆している。したがって、HIV-1 Env V3配列の荷電量が+7のgp120は、+3のgp120よりもCD4結合部位が広いことが示された。

(2) HIV-1 Env gp120の多様性解析

HIV-1 Env gp120の多様性をShannonの式を用いたエントロピー解析により調べた。V3配列の荷電量が+3では、gp120のV4およびV4周辺のみアミノ酸は多様である。一方、荷電量が+7では、V4およびV4周辺に加えて、CD4結合ループ周辺のアミノ酸がより多様である。したがって、多様性解析により、荷電量が+7では+3よりもCD4結合ループ周辺のアミノ酸がより多様であることが判明した。

D. 考察

HIV-1 Env gp120コアが同じアミノ酸配列であっても、V3配列の荷電量によってV3の配置が異なった。これはV3配列の荷電量によりV3の配置は制御されることを意味する。一方、感染者体内で比較的均一は集団として維持されるV3配列は、他の細胞指向性V3と比べて塩基性アミノ酸が少ないという特徴を持つ。ゆえにV3の配置が抗V3抗体中和を逃避する要因の一つであると考えられる。

V3配列の荷電量はV3の配置だけではなく、CD4結合ループの構造にも影響を与えた。V3配列の荷電量が大きくなるとCD4結合部位が広がった。この結果により、荷電量が大きいとCD4結合部位を認識する中和抗体による感受性が大きいと考えられる。多様性解析結果も、CD4結合ループ周辺のアミノ酸が抗体による選択圧をうけていることを示唆しており、上記の結果を支持している。

E. 結論

本研究により、V3の荷電量の低下によってgp120コアのアミノ酸配列が同じであっても、V3配列の荷電量によってV3の配置が変わることが示された。また、V3配列の荷電量はV3の配置だけではなく、gp120コアの構造にも影響を与えた。したがって、HIV-1 Env gp120 V3は荷電量に基づきウイルスの中和感受性と細胞指向性を司る機能領域と考えられる。

F. 研究発表

1 論文発表

- (1) Onyango C, Leligdowicz A, Yokoyama M, Sato H, Song H, Nakayama EE, Shioda T, de Silva T, Townend J, Jaye A, Whittle H, Rowland-Jones S, Cotton M. HIV-2 Capsids Distinguish High and Low Virus Load Patients in a West African Community Cohort. *Vaccine*, in press
- (2) Yokoyama M, Mori H, Sato H. Allosteric Regulation of HIV-1 Reverse Transcriptase by ATP for Nucleotide Selection. *PLoS ONE* 5(1): e8867, 2010.

2 学会発表

- (1) 横山 勝、長縄 聡、北村勝彦、佐藤裕徳. HIV-1 エンベロープ蛋白質の荷電変化によるウイルス中和感受性と細胞指向性の調節. 第9回日本蛋白質科学会年会、熊本、5/22/2009.
- (2) 野間口雅子、土肥直哉、藤原佐知、三宅在子、横山 勝、大出裕高、佐藤裕徳、足立昭夫. HIV-1 Envの1アミノ酸変異による増殖促進機構の解析. 第57回日本ウイルス学会学術集会、東京、10/26/2009.
- (3) 大出裕高、横山 勝、蜂谷敦子、神田忠仁、鴻永博之、佐藤裕徳. HIV-1のEFV/NVP耐性獲得とETVの抗HIV活性維持の分子機序. 第57回日本ウイルス学会学術集会、東京、10/26/2009.
- (4) 横山 勝、大出裕高、野間口雅子、神田忠仁、足立昭夫、佐藤裕徳. HIV-1 Env V3ループ構造の安定性を制御するアミノ酸. 第57回日本ウイルス学会学術集会、東京、10/27/2009.
- (5) 大出裕高、横山 勝、神田忠仁、佐藤裕徳. HIV-1前駆体蛋白質の切断効率を制御する構造特性. 第57回日本ウイルス学会学術集会、東京、10/27/2009.
- (6) 大出裕高、横山 勝、神田忠仁、佐藤裕徳、伊部史朗、藤崎誠一郎、間宮均人、濱口元洋、杉浦互、横幕能行. コンピュータシミュレーションによるHIV-1プロテアーゼの薬剤耐性度予測. 第47回日本生物物理学会年会、徳島、10/31/2009.

- (7) 大出裕高、横山 勝、蜂谷敦子、岡慎一、神田忠仁、鴻永博之、佐藤裕徳. HIV-1逆転写酵素V106I/V179D変異によるNNRTI活性変化の分子機序. 第23回日本エイズ学会学術集会・総会、名古屋、11/26/2009.
- (8) 大出裕高、横山 勝、神田忠仁、佐藤裕徳. HIV-1前駆体蛋白質切断部位の構造特性と切断効率の関連. 第23回日本エイズ学会学術集会・総会、名古屋、11/26/2009.
- (9) 横山 勝、大出裕高、野間口雅子、神田忠仁、足立昭夫、佐藤裕徳. HIV-1 Env V3ループ構造の安定性を制御するアミノ酸. 第23回日本エイズ学会学術集会・総会、名古屋、11/26/2009.
- (10) 横山 勝. 計算科学によるHIV-1 Gp120の構造解析. 第23回日本エイズ学会学術集会・総会、シンポジウム、名古屋、11/27/2009.

- (11) 野間口雅子、土肥直哉、藤原佐知、三宅在子、横山 勝、大出裕高、佐藤裕徳、足立昭夫. サル細胞指向性HIV-1 Envの細胞馴化による増殖適応変異の解析. 第23回日本エイズ学会学術集会・総会、名古屋、11/28/2009.

G. 知的財産権の出願・登録状況 (予定を含む。)

- 1 特許取得
なし。
- 2 実用新案登録
なし。
- 3 その他
なし。

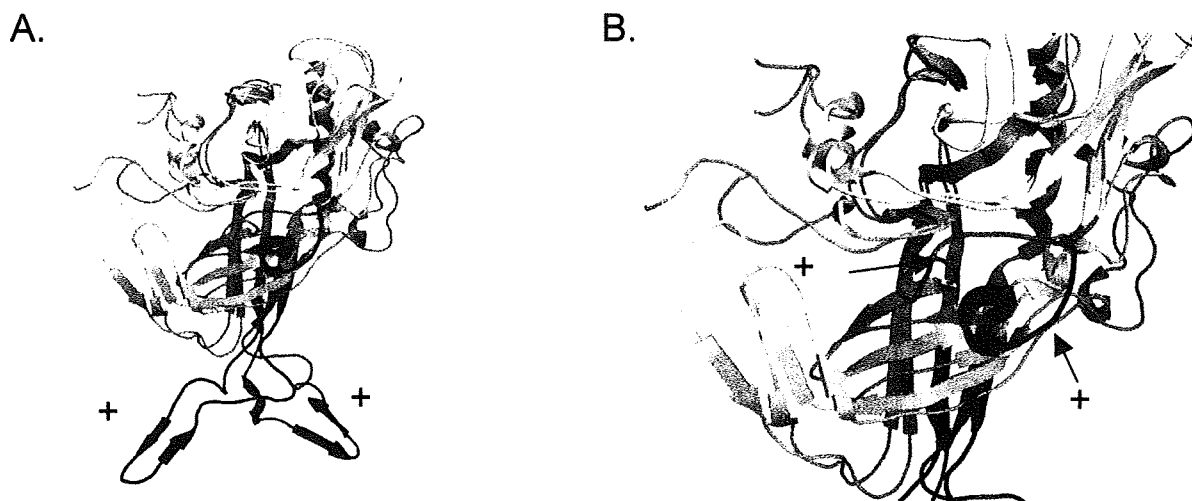


図1. HIV-1 gp120 のV3 と CD4 結合ループの配置。

ホモロジーモデリング法および分子動力学計算により構築した HIV-1 gp120 分子モデル。HIV-1 p120 outer domain 全体 (A) と CD4 結合部位周辺 (B)。V3 配列の荷電量が+3、荷電量が+7 の構造を重ね合わせている。リボン表示は gp120 を表している。+3 は V3 配列の荷電量が+3、+7 は荷電量が+7 を表す。

Ⅲ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Yamamoto T, Iwamoto N, Yamamoto H, Tsukamoto T, Kuwano T, Takeda A, Kawada M, Tsunetsugu-Yokota Y, <u>Matano T</u>	Polyfunctional CD4 ⁺ T-cell induction in neutralizing antibody-triggered control of simian immunodeficiency virus infection	J Virol	83	5514-5524	2009
Tsukamoto T, Takeda A, Yamamoto T, Yamamoto H, Kawada M, <u>Matano T</u>	Impact of cytotoxic-T-lymphocyte memory induction without virus-specific CD4 ⁺ T-cell help on control of a simian immunodeficiency virus challenge in rhesus macaques	J Virol	83	9339-9346	2009
野村拓志、 <u>俣野哲朗</u>	エイズワクチン開発の論理	ウイルス	59	267-276	2009
Yamamoto H, <u>Matano T</u>	Neutralizing antibodies in SIV control: co-impact with T cells	Vaccine		in press	
Morioka T, Yamanaka K, Mori H, Omoto Y, Tokime K, Kakeda M, Kurokawa I, Gabazza E, Tsubura A, <u>Yasutomi Y</u> , Mizutani H	IL-4/IL-13 antagonist DNA vaccination successfully suppresses Th2 type chronic dermatitis	Br J Dermatol	160	1172-1179	2009
Takano JI, Tachibana H, Kato M, Narita T, Yanagi T, <u>Yasutomi Y</u> , Fujimoto K	DNA characterization of simian Entamoeba histolytica-like strains to differentiate them from Entamoeba histolytica	Parasitol Res	105	929-937	2009
<u>Yasutomi Y</u>	Establishment of specific pathogen-free macaque colonies in Tsukuba Primate Research Center of Japan for AIDS research	Vaccine		in press	
Fujimoto K, Takano J, Narita T, Hanari K, Shimozawa N, Sankai T, Yoshida T, Terao K, Kurata T, <u>Yasutomi Y</u>	Simian Retrovirus type D infection in a colony of cynomolgus monkeys	Comp Med		in press	
Cueno ME, Karamatsu K, <u>Yasutomi Y</u> , Laurena AC, Okamoto T	Preferential expression and immunogenicity of HIV-1 Tat fusion protein expressed in tomato plant	Transgenic Res		in press	
Oka Y, Tashiro H, Mizutani-Noguchi M, Koga I, Sugao Y, Shirasaki R, <u>Miura T</u> , Akiyama N, Kawasugi K, Fujimori S, Shirafuji N	Successful unrelated bone marrow transplantation for a human immunodeficiency virus type-1-seropositive acute myelogenous leukemia patient following HAART	Int J Hematol	91	140-145	2010

Pereyra F, Palmer S, <u>Miura T</u> , Block B, Wiegand A, Rothchild A, Baker B, Rosenberg R, Cutrell E, Seaman M, Coffin J, Walker B	Persistent low level viremia in HIV-1 elite controllers and relationship to immunologic parameters	J Infect Dis	200	984-990	2009
Chen H, Piechocka-Trocha A, <u>Miura T</u> , Brockman M, Julg B, Baker B, Rothchild A, Block B, Schneidewind A, Koibuchi T, Pereyra F, Allen T, Walker B	Differential neutralization of human immunodeficiency virus (HIV) replication in autologous CD4 T cells by HIV-specific cytotoxic T lymphocytes	J Virol	83	3138-3149	2009
<u>Miura T</u> , Brumme C, Brockman M, Brumme Z, Pereyra F, Block B, Trocha A, John M, Mallal S, Harrigan PR, Walker B	HLA-associated viral mutations are common in human immunodeficiency virus type 1 elite controllers	J Virol	83	3407-3412	2009
<u>三浦聡之</u>	HIV Elite Controllers -HIV 感染症の自然制御-	感染症	39	219-223	2009
Koga M, Tachikawa A, Heckerman D, Odawara T, Nakamura H, Koibuchi T, Fujii T, <u>Miura T</u> , Iwamoto A	Transition of impact of HLA class I allele expression on HIV-1 plasma virus loads at a population level over time	Microbiol Immunol		in press	
Nishi M, Ryo A, Tsurutani N, Ohba K, Sawasaki T, Morishita R, Perrem K, Aoki I, <u>Morikawa Y</u> , Yamamoto N	Requirement for microtubule integrity in the SOCS1-mediated intracellular dynamics of HIV-1 Gag	FEBS Let	585	1243-1250	2009
Suyama M, Daikoku E, Goto T, Sano K, <u>Morikawa Y</u>	Reactivation from latency displays HIV particle budding at plasma membrane, accompanying CD44 upregulation and recruitment	Retrovirology	6	63	2009
Urano E, Ichikawa R, <u>Morikawa Y</u> , Yoshida T, Koyanagi Y, Komano J	T cell-based functional cDNA library screening identified SEC13-like 1a carboxy-terminal domain as a negative regulator of human immunodeficiency virus replication	Vaccine		in press	
<u>Yokoyama M</u> , Mori H, Sato H	Allosteric regulation of HIV-1 reverse transcriptase by ATP for nucleotide selection	PLoS ONE	5	e8867	2010
Onyango C, Leligdowicz A, <u>Yokoyama M</u> , Sato H, Song H, Nakayama EE, Shioda T, de Silva T, Townend J, Jaye A, Whittle H, Rowland-Jones S, Cotton M	HIV-2 capsids distinguish high and low virus load patients in a West African community cohort	Vaccine		in press	

IV. 研究成果の刊行物・別刷

Polyfunctional CD4⁺ T-Cell Induction in Neutralizing Antibody-Triggered Control of Simian Immunodeficiency Virus Infection[∇]

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Received 22 January 2009/Accepted 6 March 2009

Rapid depletion of memory CD4⁺ T cells and delayed induction of neutralizing antibody (NAb) responses are characteristics of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) infections. Although it was speculated that postinfection NAb induction could have only a limited suppressive effect on primary HIV replication, a recent study has shown that a single passive NAb immunization of rhesus macaques 1 week after SIV challenge can result in reduction of viral loads at the set point, indicating a possible contribution of postinfection NAb responses to virus control. However, the mechanism accounting for this NAb-triggered SIV control has remained unclear. Here, we report rapid induction of virus-specific polyfunctional T-cell responses after the passive NAb immunization postinfection. Analysis of SIV Gag-specific responses of gamma interferon, tumor necrosis factor alpha, interleukin-2, macrophage inflammatory protein 1β, and CD107a revealed that the polyfunctionality of Gag-specific CD4⁺ T cells, as defined by the multiplicity of these responses, was markedly elevated in the acute phase in NAb-immunized animals. In the chronic phase, despite the absence of detectable NAb, virus control was maintained, accompanied by polyfunctional Gag-specific T-cell responses. These results implicate virus-specific polyfunctional CD4⁺ T-cell responses in this NAb-triggered virus control, suggesting possible synergism between NAb and T cells for control of HIV/SIV replication.

Virus-specific CD4⁺ and CD8⁺ T-cell responses are crucial for the control of pathogenic human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV) infections (5, 6, 20, 23, 30, 39, 40). However, CD4⁺ T cells, especially CCR5⁺ memory CD4⁺ T cells, are themselves targets for these viruses, which may be an obstacle to potent virus-specific CD4⁺ T-cell induction (10, 47, 52). Indeed, HIV-1/SIV infection causes rapid, massive depletion of memory CD4⁺ T cells (26, 31), and host immune responses fail to contain viral replication and allow persistent chronic infection, although virus-specific CD8⁺ T-cell responses exert suppressive pressure on viral replication (15).

Recently, the importance of T-cell quality in virus containment has been high-lighted, and T-cell polyfunctionality, which is defined by their multiplicity of antigen-specific cytokine production, has been analyzed as an indicator of T-cell quality (4, 8, 11, 41). However, there has been no evidence indicating an association of polyfunctional T-cell responses in the acute phase with HIV-1/SIV control. Even in the chronic phase, whether polyfunctional CD4⁺ T-cell responses may be associ-

ated with virus control has been unclear, although an inverse correlation between polyfunctional CD8⁺ T-cell responses and viral loads has been shown in HIV-1-infected individuals (4).

Another characteristic of HIV-1/SIV infections is the absence of potent neutralizing antibody (NAb) induction during the acute phase (7). This is mainly due to the unusually neutralization-resistant nature of the virus, such as masking of target epitopes in viral envelope proteins (24). Whether this lack of effective NAb response contributes to the failure to control the virus, and whether NAb induction in the acute phase can contribute to virus control, remains unclear. Previous studies documenting virus escape from NAb recognition suggested that NAb can also exert selective pressure on viral replication to a certain extent (38, 45, 49), but it was speculated that postinfection NAb induction could have only a limited suppressive effect on primary HIV-1/SIV replication (34, 37).

By passive NAb immunization of rhesus macaques after SIV challenge, we recently provided evidence indicating that the presence of NAb during the acute phase can result in SIV control (50). In that study, passive NAb immunization 1 week after SIVmac239 challenge resulted in transient detectable NAb responses followed by reduction in set point viral loads compared to unimmunized macaques. However, the mechanism of this virus control has remained unclear. In the present study, we found rapid appearance of polyfunctional Gag-specific CD4⁺ T-cell responses after such passive NAb immunization postinfection. These animals maintained virus control

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[∇] Published ahead of print on 18 March 2009.

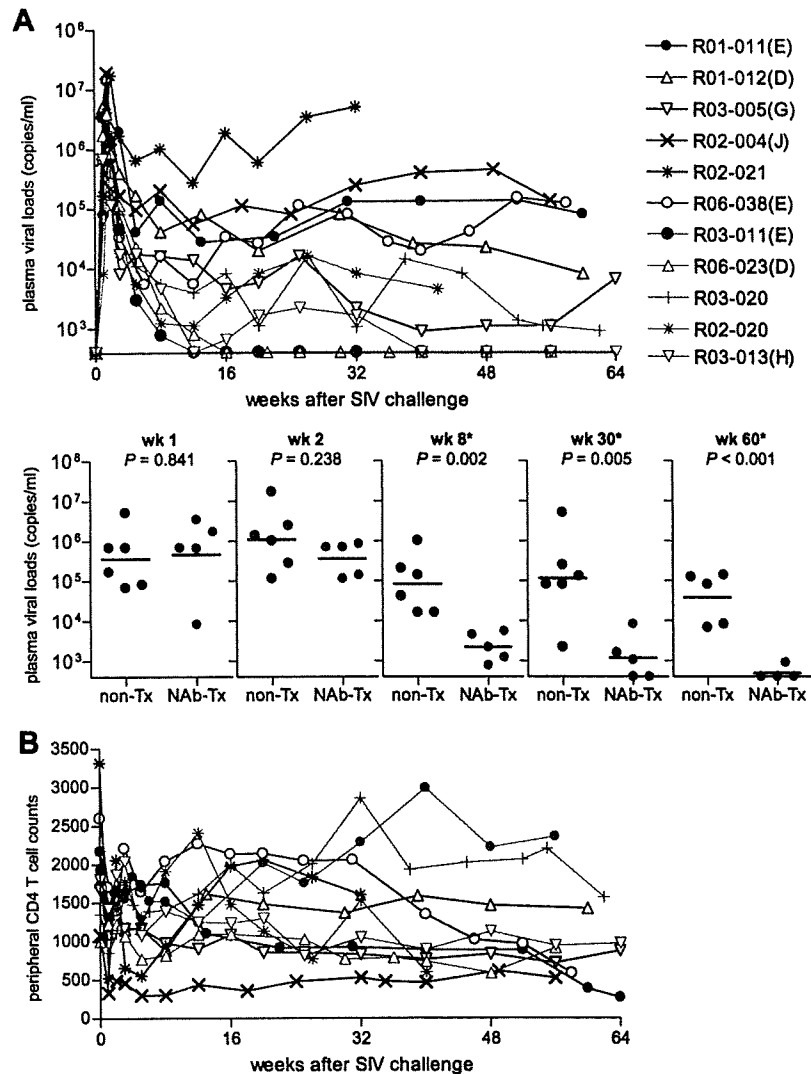


FIG. 1. Follow up of NAb-immunized macaques. (A) Plasma viral loads (SIV *gag* RNA copies/ml plasma) in six unimmunized macaques (black lines) and five NAb-immunized animals (red lines) after SIVmac239 challenge. The plasma viral loads were measured as described previously (29). The lower limit of detection was approximately 4×10^2 copies/ml. The MHC-I haplotypes are shown in parentheses following the macaque numbers as follows: E, haplotype 90-010-Ie; D, 90-010-Id; G, 90-030-Ig; J, 90-088-Ij; and H, 90-030-Ih. Below are comparisons of plasma viral loads in unimmunized (non-Tx) and NAb-immunized (NAb-Tx) macaques at weeks (wk) 1, 2, 8, 30, and 60. The bars indicate the geometric mean of each group. The comparisons at weeks 8, 30, and 60 (indicated by asterisks) showed significant differences between two groups ($P = 0.841$ at week 1, $P = 0.238$ at week 2, $P = 0.002$ at week 8, $P = 0.005$ around week 30, and $P < 0.001$ around week 60 by *t* test). (B) Peripheral CD4⁺ T-cell counts (per μ l) in unimmunized controls (black lines) and NAb-immunized macaques (red lines) after SIVmac239 challenge. The ratios of the counts around week 60 to those at week 0 in NAb-immunized macaques were significantly higher than in unimmunized controls ($P = 0.028$ by *t* test).

for more than 1 year in the absence of detectable plasma NAbs, which was accompanied by potent Gag-specific T-cell responses. These results implicate virus-specific polyfunctional CD4⁺ T-cell responses in this NAb-triggered primary and long-term SIV control.

MATERIALS AND METHODS

Animal experiments. We previously showed a reduction in set point viral loads by passive NAb immunization of rhesus macaques (*Macaca mulatta*) 1 week after SIVmac239 challenge (50). In the present study, we monitored these animals, including one additional NAb-immunized macaque (R06-023), for more than 1

year. Thus, five NAb-immunized rhesus macaques and six unimmunized controls were used in this study. Unimmunized macaque R02-021 was euthanized at week 32 because the animal showed loss of body weight, diarrhea, and general weakness. NAb-immunized macaque R02-020 was euthanized at week 42 because of a limitation on available cage numbers. Major histocompatibility complex class I (MHC-I) haplotypes were determined by reference strand-mediated conformation analysis as described previously (2, 29). A group of rhesus macaques possessing the MHC-I haplotype 90-120-Ia with the potential to efficiently elicit potent Gag-specific CD8⁺ T-cell responses (21, 29) were not included in this study. All animals were maintained in accordance with the guidelines for animal experiments performed at the National Institute of Infectious Diseases (33).

For passive NAb immunization, immunoglobulin G (IgG) was purified from plasma samples from SIVmac239-infected macaques with SIV-specific NAb re-

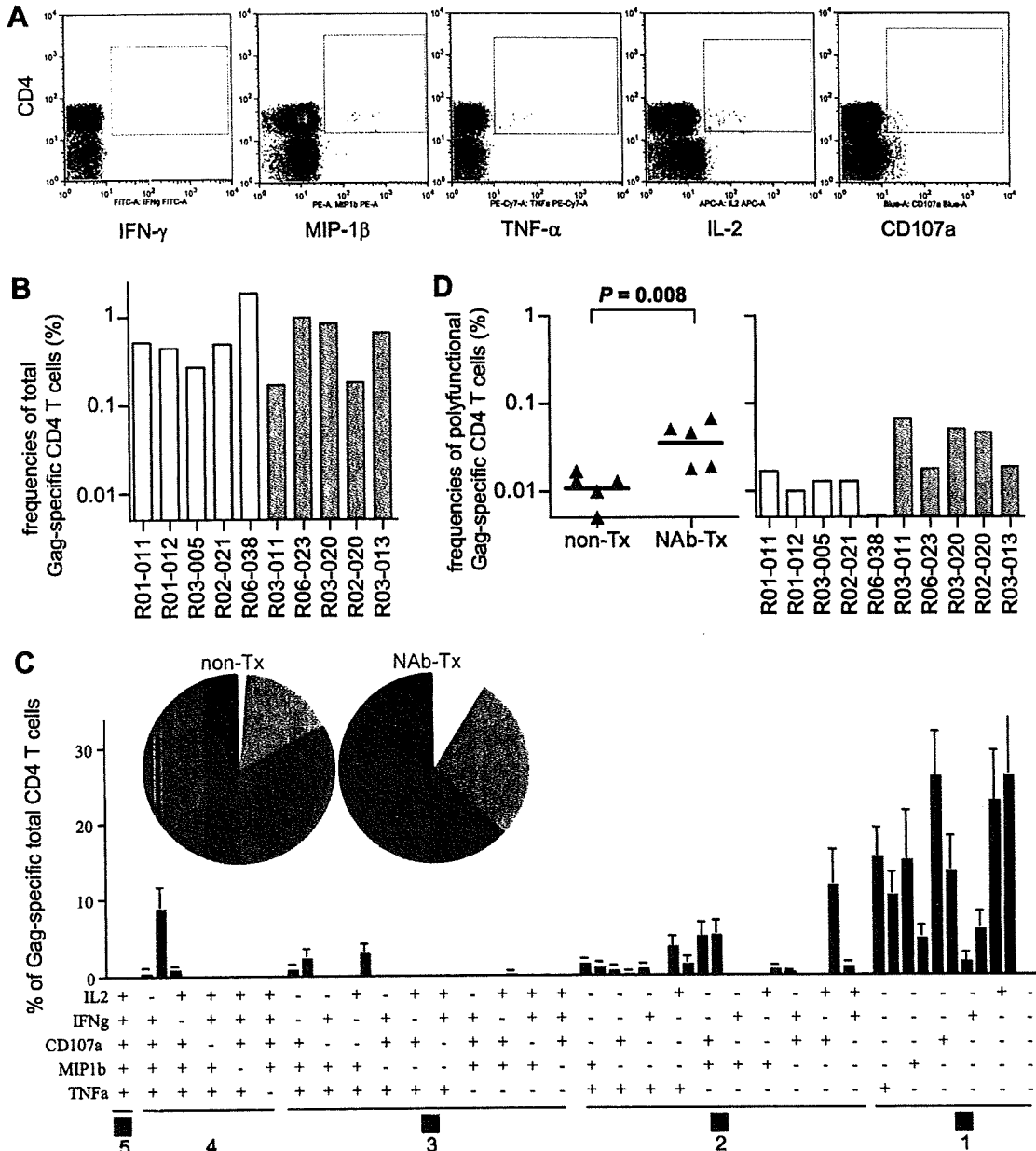


FIG. 2. Gag-specific CD4⁺ T-cell responses in the acute phase. PBMCs at week 2 postchallenge were stimulated with a recombinant SIV Gag p55, and the specific induction of five marker cytokines (IFN- γ , TNF- α , IL-2, MIP-1 β , and CD107a) in CD4⁺ T cells was examined. PBMCs from macaque R02-004 were unavailable and therefore could not be included in this analysis. (A) Representative dot plots showing responses of IFN- γ , TNF- α , IL-2, MIP-1 β , and CD107a in CD4⁺ T cells in macaque R03-013 after Gag-specific stimulation. Gag-specific IFN- γ responses were undetectable at week 2 in this animal. (B) Frequencies of total Gag-specific CD4⁺ T cells that showed Gag-specific induction of IFN- γ , TNF- α , IL-2, MIP-1 β , or CD107a in total CD4⁺ T cells. (C) Percentages of cells exhibiting Gag-specific induction of single or multiple marker cytokines in total Gag-specific CD4⁺ T cells. (Top) Mean percentages of Gag-specific CD4⁺ T cells producing one (dark blue), two (light blue), three (green), four (yellow), or five (red) marker cytokines in unimmunized (non-Tx) and NAb-immunized (NAb-Tx) macaques. (Bottom) Mean percentages of individual Gag-specific CD4⁺ T-cell subsets divided by the patterns of marker cytokine induction in unimmunized (blue bars) and NAb-immunized (red bars) macaques. (D) Frequencies of polyfunctional Gag-specific CD4⁺ T cells that showed Gag-specific induction of ≥ 3 marker cytokines in total CD4⁺ T cells. On the left, the bars indicate the geometric mean of each group. The frequencies in NAb-immunized macaques ($n = 5$) were significantly higher than in unimmunized controls ($n = 5$) ($P = 0.008$).

sponses in the chronic phase, as described previously (50). The SIVmac239-specific NAb titer of this IgG preparation (30 mg/ml) was 1:16 on MT-4 cells. Animals were intravenously infused with 300 mg of IgG 1 week after challenge with 1,000 50% tissue culture infective doses (TCID₅₀) of SIVmac239 (22). Two unimmunized controls, R02-021 and R06-038, received 300 mg of control IgG

prepared from noninfected rhesus macaques at week 1. Neutralizing titers were measured as described previously (50). In brief, serial twofold dilutions of heat-inactivated plasma in duplicate were incubated with 10 TCID₅₀ of SIVmac239 for 45 min (5 μ l of diluted sample was incubated with 5 μ l of virus in each mixture) and added to 5 \times 10⁴ MT-4 cells/well in 96-well plates. Progeny virus

production in day 12 culture supernatants was examined by enzyme-linked immunosorbent assays for detection of SIV p27 core antigen (Advanced BioScience Laboratories, Inc., Kensington, MD) to determine the 100% neutralizing end-point. The lower limit of titration was 1:2.

Analysis of polyfunctional Gag-specific T-cell responses. We analyzed Gag-specific induction of gamma interferon (IFN- γ), tumor necrosis factor alpha (TNF- α), interleukin-2 (IL-2), macrophage inflammatory protein 1 β (MIP-1 β), and CD107a in CD4 $^{+}$ and CD8 $^{+}$ T cells as described previously (1). Peripheral blood mononuclear cells (PBMCs) were cultured for 6 h in the absence or the presence of 10 μ g/ml of a recombinant SIV Gag p55 (Protein Sciences, Meriden, CT) for unstimulated controls or Gag-specific stimulation (12). They were incubated with anti-human CD28 and anti-human CD49d antibodies (5 μ g/ml) (BD, Tokyo, Japan) for costimulation and with anti-human CD107a antibody (BD) for immunostaining. Monensin (BD) and brefeldin A (Sigma-Aldrich, Tokyo, Japan) were added to the culture for the final 5 h of stimulation. Then, immunostaining was performed using a Cytofix/Cytoperm kit (BD) and the following monoclonal antibodies: fluorescein isothiocyanate-conjugated anti-human IFN- γ (BD), phycoerythrin (PE)-conjugated anti-human MIP-1 β (BD), peridinin chlorophyll protein-conjugated anti-human CD4 (BD), allophycocyanin (APC)-conjugated anti-human IL-2 (BD), PE-Cy7-conjugated anti-human TNF- α (BD), APC-Cy7-conjugated anti-human CD3 (BD), energy-coupled dye-conjugated anti-human CD69 (Beckman Coulter, Tokyo, Japan), biotin-conjugated anti-human CD8 (BD), and anti-human CD107a (BD) conjugated with Pacific Blue using a Zeon mouse IgG1 labeling kit (Invitrogen, Tokyo, Japan). Flow-cytometric 10-color analysis of the induction of the five marker cytokines, IFN- γ , TNF- α , IL-2, MIP-1 β , and CD107a, was performed using the FACSAria system (BD); 3×10^5 to 5×10^5 lymphocyte events were analyzed. The data were analyzed using FlowJo (version 8.2; TreeStar Inc., Ashland, OR) and FACSDiva (BD) software. Analysis of polyfunctional phenotypes of T cells was carried out using PESTLE (version 1.5.4) and SPICE (version 4.1.6) programs, which were generously provided by Mario Roederer (National Institutes of Health, Bethesda, MD). Specific T-cell levels were calculated by subtracting nonspecific T-cell frequencies from those after Gag-specific stimulation. Specific T-cell levels of less than 0.01% were considered negative.

Analysis of proliferative Gag-specific CD4 $^{+}$ T-cell responses. Gag-specific CD4 $^{+}$ T-cell proliferation was assessed by bromodeoxyuridine (BrdU) incorporation as described previously (9). In brief, PBMCs were cultured in the absence or the presence of 10 μ g/ml p55 for 6 days for unstimulated controls or Gag-specific stimulation. Then, the cells were incubated for 2 h with 100 ng/ml BrdU and immunostained using the following monoclonal antibodies: peridinin chlorophyll protein-conjugated anti-human CD4, APC-conjugated anti-human CD95 (BD), APC-Cy7-conjugated anti-human CD3, and energy-coupled dye-conjugated anti-human CD28 (Beckman Coulter, Tokyo, Japan) for surface staining and fluorescein isothiocyanate-labeled anti-human BrdU (BD) for intracellular staining. As a positive control, PBMCs were stimulated with 1 μ g/ml staphylococcal enterotoxin B for 3 days. Flow-cytometric analysis was performed using the FACSAria system, and the data were analyzed using FlowJo (version 8.2).

In vitro viral suppression assay. We examined SIVmac239 replication on CD8-depleted PBMCs in the presence of CD8 $^{+}$ cells positively selected from PBMCs as described previously (46). In brief, PBMCs were separated into CD8 $^{+}$ cells and CD8 $^{-}$ cells by using Macs CD8 MicroBeads (Miltenyi Biotec, Tokyo, Japan). For preparing target cells, the CD8 $^{-}$ cells negatively selected from PBMCs obtained before challenge were infected with SIVmac239 at a multiplicity of infection of $1:10^4$ TCID $_{50}$ /cell and cultured in the presence of 2 μ g/ml phytohemagglutinin L (Roche Diagnostics) and 20 IU/ml recombinant human IL-2 (Roche Diagnostics). Two days later, effector CD8 $^{+}$ cells positively selected from PBMCs obtained before challenge or at week 3 or 4 were added to the target cells at an effector/target (E/T) ratio of 1:4. The culture supernatants were harvested every other day. Reverse transcriptase activities in these supernatants were measured to confirm the peak of viral production in the control culture of target cells without CD8 $^{+}$ cells around day 10 after SIV infection. SIV Gag capsid protein p27 concentrations in the supernatants after 8 days of coculture (i.e., at day 10 after SIV infection) were then measured by enzyme-linked immunosorbent assay. Results from macaques R01-011, R03-005, R02-021, and R06-023 were excluded because mean p27 concentrations in the control cultures without CD8 $^{+}$ cells or in one of the duplicates were less than 50 ng/ml. The lower limit of p27 detection was approximately 0.2 ng/ml.

Statistical analysis. Statistical analysis was performed with 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 and specific T-cell frequencies were log transformed and compared between unimmunized controls and NAb-immunized macaques by an unpaired two-tailed t test. Correlation was analyzed by the Pearson test.

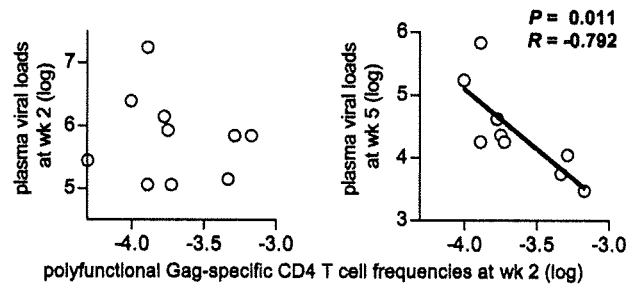


FIG. 3. Analysis of correlation between polyfunctional Gag-specific CD4 $^{+}$ T-cell frequencies (log) at week (wk) 2 and plasma viral loads (log) at week 2 (left) or week 5 (right). Inverse correlation is shown on the right ($P = 0.011$; $R = -0.792$), but not on the left ($P = 0.694$; $R = -0.143$).

RESULTS

Long-term SIV control after passive NAb immunization postinfection. In order to evaluate the long-term effect on SIV replication of a single passive NAb immunization in the acute phase, we monitored animals for more than 1 year after SIVmac239 challenge (Fig. 1). Five NAb-immunized rhesus macaques and six unimmunized controls, including two animals that received control antibodies at week 1, were followed up. Of these, NAb-immunized macaque R03-011 and two unimmunized controls, R01-011 and R06-038, shared the MHC-I haplotype *90-010-Ie*, and NAb-immunized R06-023 and unimmunized R01-012 shared *90-010-Id*. We previously reported that a group of Burmese rhesus macaques possessing the MHC-I haplotype *90-120-Ia* mounted efficient Gag-specific CD8 $^{+}$ T-cell responses and showed vaccine-based SIV control (21, 29), but those animals were not included in the present study.

The plasma viral loads of both NAb-immunized and unimmunized macaques were similar at week 1, just before NAb administration (Fig. 1A). At week 2 postchallenge, i.e., 1 week after NAb administration, the geometric mean of plasma viral loads in NAb-immunized macaques was slightly lower than in unimmunized controls, but this difference did not achieve statistical significance. At week 8, however, the difference became significant, with lower plasma viral loads in NAb-immunized animals (Fig. 1A). Thereafter, the NAb-immunized macaques maintained significantly reduced viral loads for more than 1 year. In the chronic phase, plasma viral loads were less than 1×10^4 copies/ml in all five NAb-immunized macaques and were even undetectable in three of them. NAb-immunized macaque R03-011, possessing the MHC-I haplotype *90-010-Ie*, contained SIV replication with undetectable viremia, whereas unimmunized macaques R01-011 and R06-038, which shared this haplotype, had high viral loads. The NAb-immunized macaque R06-023, with MHC-I haplotype *90-010-Id*, contained SIV replication, whereas unimmunized macaque R01-012, which shared the same haplotype, failed to control viremia. Peripheral CD4 $^{+}$ T-cell counts were maintained in the NAb-immunized macaques during the observation period (Fig. 1B).

We examined SIVmac239-specific neutralizing antibody responses by determining the end point plasma titers for inhibiting 10-TCID $_{50}$ virus replication on MT-4 cells (data not shown). In NAb-immunized macaques, NAb responses were

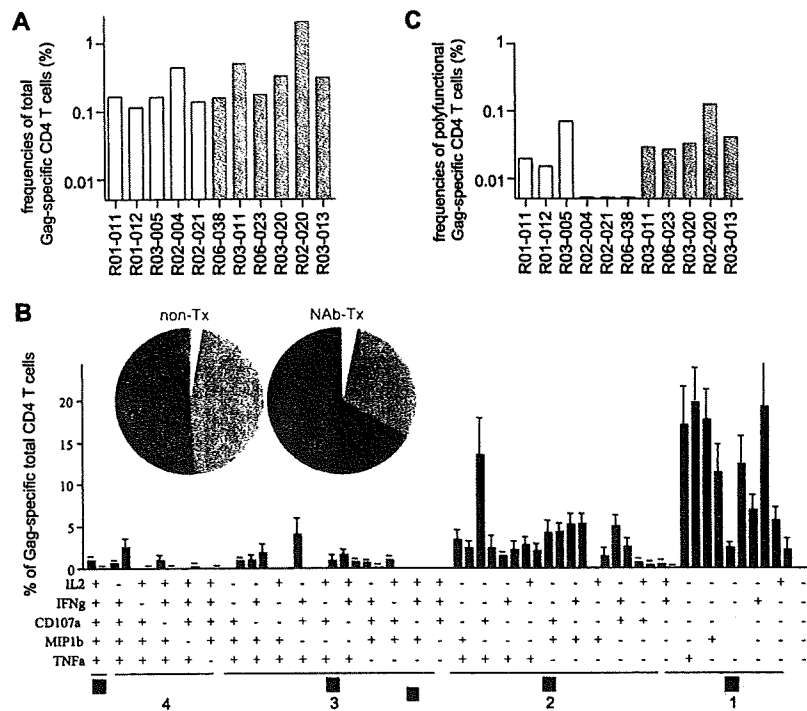


FIG. 4. Gag-specific CD4⁺ T-cell responses in the chronic phase. PBMCs around week 30 postchallenge were stimulated with p55, and specific induction of IFN- γ , TNF- α , IL-2, MIP-1 β , and CD107a in CD4⁺ T cells was examined. (A) Frequencies of total Gag-specific CD4⁺ T cells. (B) Percentages of cells exhibiting Gag-specific induction of single or multiple marker cytokines in total Gag-specific CD4⁺ T cells. See the legend to Fig. 2 for symbols. (C) Frequencies of polyfunctional Gag-specific CD4⁺ T cells exhibiting Gag-specific induction of ≥ 3 marker cytokines in total CD4⁺ T cells. The frequencies in NAb-immunized macaques ($n = 5$) were significantly higher than in unimmunized controls ($n = 6$) ($P = 0.046$).

detected at day 10 postchallenge but became undetectable within 1 week of passive NAb immunization, as described previously (50), implying that the infused NABs were rapidly exhausted for virus clearance. None of the animals had detectable de novo NAB responses even around week 40 after challenge. In unimmunized controls, SIVmac239-specific NAB responses were also undetectable, except in one animal, R01-012, after week 30. Thus, passive NAB immunization 1 week after SIV challenge resulted in a transient period of NAB

detection, followed by sustained virus control in the absence of detectable NAB responses.

Polyfunctional Gag-specific CD4⁺ T-cell responses in the acute phase in passively NAB-immunized macaques. To investigate whether virus-specific T-cell responses were involved in this NAB-triggered SIV control, we first analyzed SIV Gag-specific CD4⁺ T-cell responses in the acute phase. We stimulated PBMCs obtained at week 2 with a recombinant SIV Gag p55 protein and analyzed Gag-specific induction of IFN- γ ,

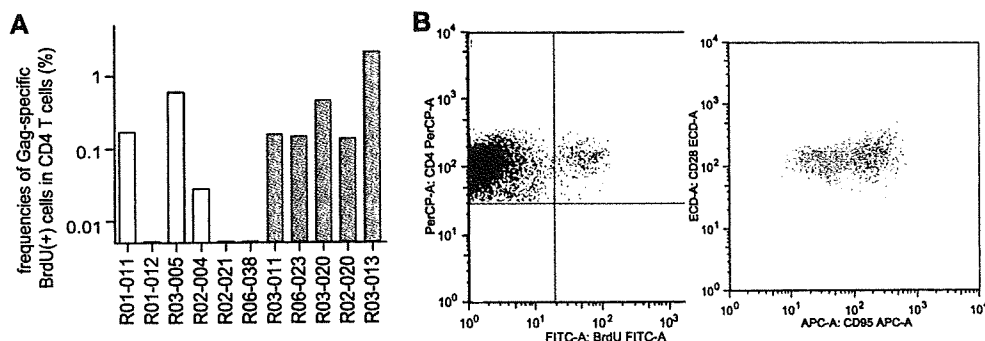


FIG. 5. Gag-specific CD4⁺ T-cell proliferative responses in the chronic phase. PBMCs around week 30 postchallenge were stimulated with p55, and specific uptake of BrdU in CD4⁺ T cells was examined. (A) Frequencies of Gag-specific BrdU⁺ CD4⁺ T cells in total CD4⁺ T cells. The frequencies in NAB-immunized macaques were significantly higher than in unimmunized controls ($P = 0.042$). (B) A representative density plot (gated on CD3⁺ lymphocytes) showing BrdU⁺ CD4⁺ T-cell induction after Gag stimulation (macaque R03-013). Most Gag-specific BrdU⁺ CD4⁺ T cells gated in the left-hand plot were CD95⁺ CD28⁺ (indicated by red) in the right-hand plot gated on CD3⁺ CD4⁺ lymphocytes. FITC, fluorescein isothiocyanate; PerCP, peridinin chlorophyll protein.

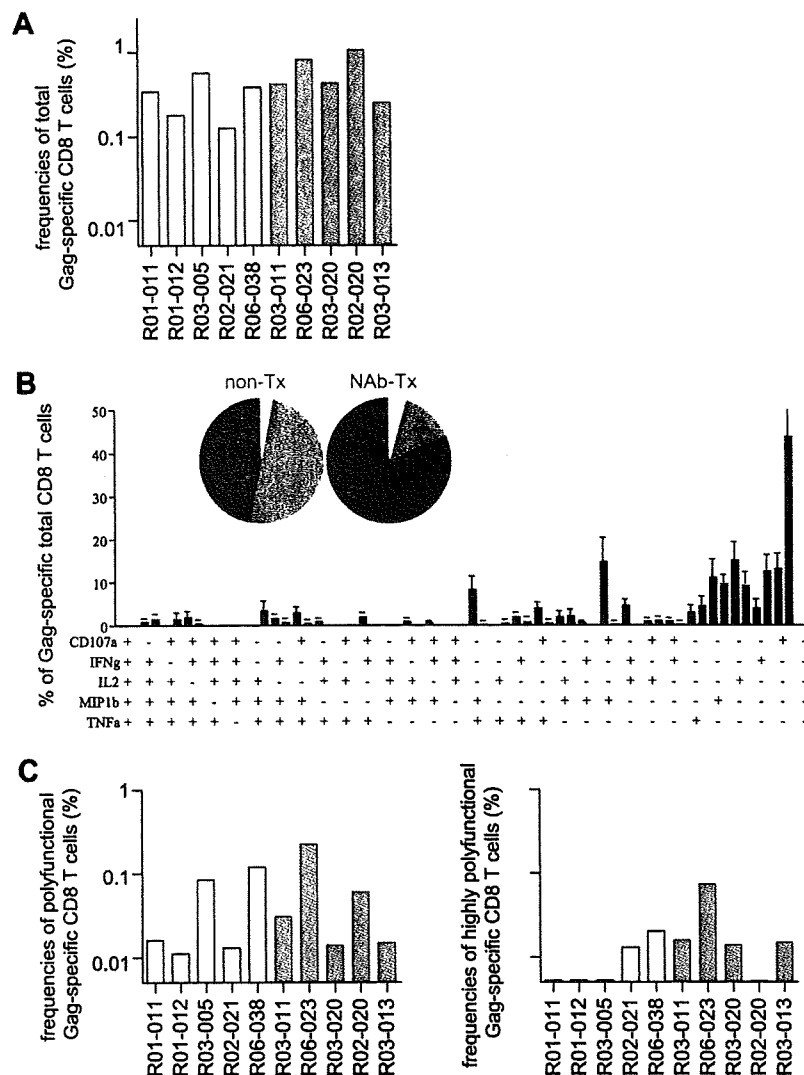


FIG. 6. Gag-specific CD8⁺ T-cell responses in the acute phase. PBMCs at week 2 were stimulated with p55, and specific induction of IFN- γ , TNF- α , IL-2, MIP-1 β , and CD107a in CD8⁺ T cells was examined. (A) Frequencies of total Gag-specific CD8⁺ T cells. (B) Percentages of cells exhibiting Gag-specific induction of single or multiple marker cytokines in total Gag-specific CD8⁺ T cells. See the legend to Fig. 2 for symbols. (C) Frequencies of Gag-specific CD8⁺ T cells exhibiting Gag-specific induction of ≥ 3 marker cytokines (polyfunctional; left) or ≥ 4 marker cytokines (highly polyfunctional; right) in total CD8⁺ T cells.

TNF- α , IL-2, and MIP-1 β and surface mobilization of CD107a (a degranulation marker) in CD4⁺ T cells (Fig. 2A) (14, 25, 41). The Gag-specific responses of each factor, IFN- γ , TNF- α , IL-2, MIP-1 β , and CD107a, in CD4⁺ T cells did not show significant differences between unimmunized and NAb-immunized animals (data not shown). We then analyzed these five factors to assess the polyfunctionality of virus-specific T cells and refer to them as marker cytokines in this study. No significant differences in the frequencies of total Gag-specific CD4⁺ T cells (i.e., CD4⁺ T cells exhibiting Gag-specific induction of one or more of the marker cytokines IFN- γ , TNF- α , IL-2, MIP-1 β , and CD107a) were observed between the two groups (Fig. 2B).

We examined the polyfunctionality of SIV Gag-specific CD4⁺ T cells, as defined by their multiplicity of marker cyto-

kines induced by Gag-specific stimulation (11, 41) (Fig. 2C). The mean percentage of cells producing ≥ 3 marker cytokines (Fig. 2C, sum of red, yellow, and green) in the Gag-specific CD4⁺ T-cell pool was more than 15% in NAb-immunized macaques but less than 3% in unimmunized controls. The frequencies of these polyfunctional Gag-specific CD4⁺ T cells within the CD4⁺ T-cell pool were significantly higher in the immunized animals, with a solid difference ($P = 0.008$ by *t* test) (Fig. 2D). Indeed, all the NAb-immunized macaques had higher frequencies of polyfunctional Gag-specific CD4⁺ T cells than any of the unimmunized controls, indicating that passive NAb immunization 1 week after SIV challenge resulted in rapid induction of Gag-specific CD4⁺ T cells with higher polyfunctionality at week 2.

The polyfunctional Gag-specific CD4⁺ T-cell frequencies at

week 2 were inversely correlated with plasma viral loads at week 5 (Fig. 3). The inverse correlation, however, was not indicated with plasma viral loads at week 2. These results implicate rapidly induced polyfunctional Gag-specific CD4⁺ T-cell responses in subsequent reduction of plasma viral loads in NAb-immunized macaques.

Polyfunctional Gag-specific CD4⁺ T-cell responses in the chronic phase in NAb-immunized macaques. We then examined SIV Gag-specific CD4⁺ T-cell responses in the chronic phase. Around week 30 after challenge, total Gag-specific CD4⁺ T-cell frequencies in NAb-immunized animals were similar to or, if anything, higher than those in unimmunized controls (Fig. 4A). The Gag-specific responses of each marker cytokine in CD4⁺ T cells showed no significant difference between the two groups (data not shown). The polyfunctionalities of these Gag-specific CD4⁺ T cells (the percentage of cells producing ≥ 3 marker cytokines) within the total Gag-specific CD4⁺ T-cell population in both groups were similar (Fig. 4B). However, the frequencies of these polyfunctional Gag-specific CD4⁺ T cells as a fraction of total CD4⁺ T cells in NAb-immunized macaques were higher than in unimmunized controls (Fig. 4C).

We also examined the SIV Gag-specific proliferative responses of CD4⁺ T cells around week 30 by measurement of BrdU uptake after Gag-specific stimulation (Fig. 5A). This revealed higher proliferative responses of Gag-specific CD4⁺ T cells in NAb-immunized macaques than in unimmunized controls. Gag-specific CD4⁺ T-cell proliferative responses were detectable in all the NAb-immunized macaques but in only three of six unimmunized controls. Most of the BrdU⁺ CD4⁺ T cells after Gag-specific stimulation were of the central memory (CD95⁺ CD28⁺) phenotype (36) (Fig. 5B). These results suggest that NAb-immunized macaques had potent Gag-specific CD4⁺ T cells with efficient proliferative ability in the chronic phase.

CD8⁺ cells with high anti-SIV efficacy in NAb-immunized macaques. The above-mentioned results revealed higher frequencies of polyfunctional Gag-specific CD4⁺ T-cell responses in NAb-immunized macaques. We next analyzed Gag-specific CD8⁺ T-cell responses in the acute phase (Fig. 6). At week 2, total Gag-specific CD8⁺ T-cell frequencies were similar, and no clear difference in frequencies of Gag-specific CD8⁺ T cells producing ≥ 3 or ≥ 4 marker cytokines was detected between the two groups.

We then examined, by *in vitro* viral-suppression assays (13, 27, 46, 51), whether the CD8⁺ cells from these NAb-immunized macaques had the potential to control SIV replication more efficiently than those from the controls (Fig. 7). In this assay, CD8⁻ target cells prepared by CD8-negative selection from PBMCs were infected with SIVmac239 and cocultured with effector CD8⁺ cells prepared by CD8-positive selection from PBMCs at week 3. We obtained results from four NAb-immunized macaques and three unimmunized controls.

Three of four NAb-immunized macaques (R03-011, R03-020, and R03-013) showed more than 100-fold reduction in viral production at an E/T ratio of 1:4, although the remaining animal (R02-020) failed to show strong anti-SIV efficacy *in vitro*. Of the NAb-immunized animals, this individual R02-020 maintained the highest viral loads in the chronic phase. In contrast to CD8⁺ cells from the majority of immunized ani-

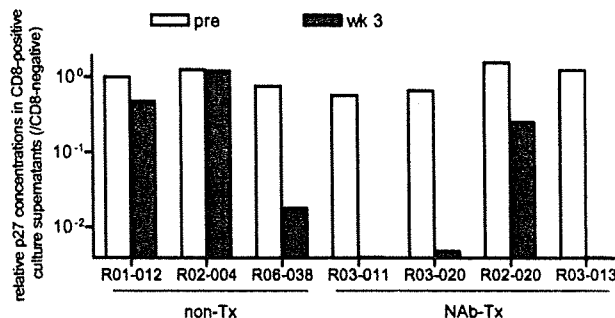


FIG. 7. Anti-SIV efficacy *in vitro* of CD8⁺ cells. PBMC-derived CD8⁻ (target) cells infected with SIVmac239 were cultured alone (no CD8) or cocultured with autologous PBMC-derived CD8⁺ (effector) cells obtained prechallenge (pre) or at week 3 postchallenge (wk 3) at an E/T ratio of 1:4. The results were obtained from three unimmunized controls and four NAb-immunized macaques. The ratios of p27 concentrations in the culture supernatants after 8 days of coculture with pre-CD8⁺ or week 3 CD8⁺ cells to those without CD8⁺ cells (CD8 negative) are shown. The coculture with either R03-011 week 3 CD8⁺, R03-020 week 3 CD8⁺, and R03-013 week 3 CD8⁺ cells showed undetectable or marginal SIV p27 production after 8 days.

mals, CD8⁺ cells from the unimmunized controls (R01-012, R02-004, and R06-038) showed weak anti-SIV efficacy. In fact, the reduction of virus production by CD8⁺ cells from the unimmunized macaques R01-012 and R06-038 was less than 100-fold even in coculture at an E/T ratio of 1:1 (data not shown; not determined for R02-004). These results suggest that passive NAb immunization may facilitate the induction of potent CD8⁺ cells possessing higher anti-SIV efficacy.

Polyfunctional Gag-specific CD8⁺ T-cell responses in the chronic phase in NAb-immunized macaques. We next examined SIV Gag-specific CD8⁺ T-cell responses in the chronic phase (Fig. 8). Around week 30 after challenge, the geometric means of total Gag-specific CD8⁺ T-cell frequencies in NAb-immunized animals were higher than in unimmunized controls, but this difference did not achieve statistical significance. In particular, NAb-immunized macaques showed significantly higher levels of Gag-specific IFN- γ responses in CD8⁺ T cells (data not shown). There was no clear difference in polyfunctional Gag-specific CD8⁺ T-cell responses between the two groups. However, highly polyfunctional Gag-specific CD8⁺ T cells producing ≥ 4 marker cytokines were detectable in all NAb-immunized macaques, and the frequencies of these highly polyfunctional Gag-specific CD8⁺ T cells in the total CD8⁺ T-cell population were higher than in unimmunized controls.

DISCUSSION

In our previous study (50), a single passive NAb immunization of rhesus macaques 1 week after SIVmac239 challenge resulted in significant reduction of set point viral loads. The present study has shown that this NAb-triggered virus control was maintained in the absence of detectable NABs in the chronic phase. Remarkably, virus-specific CD4⁺ T-cell responses with higher polyfunctionality were rapidly induced in NAb-immunized macaques. These results implicate more po-

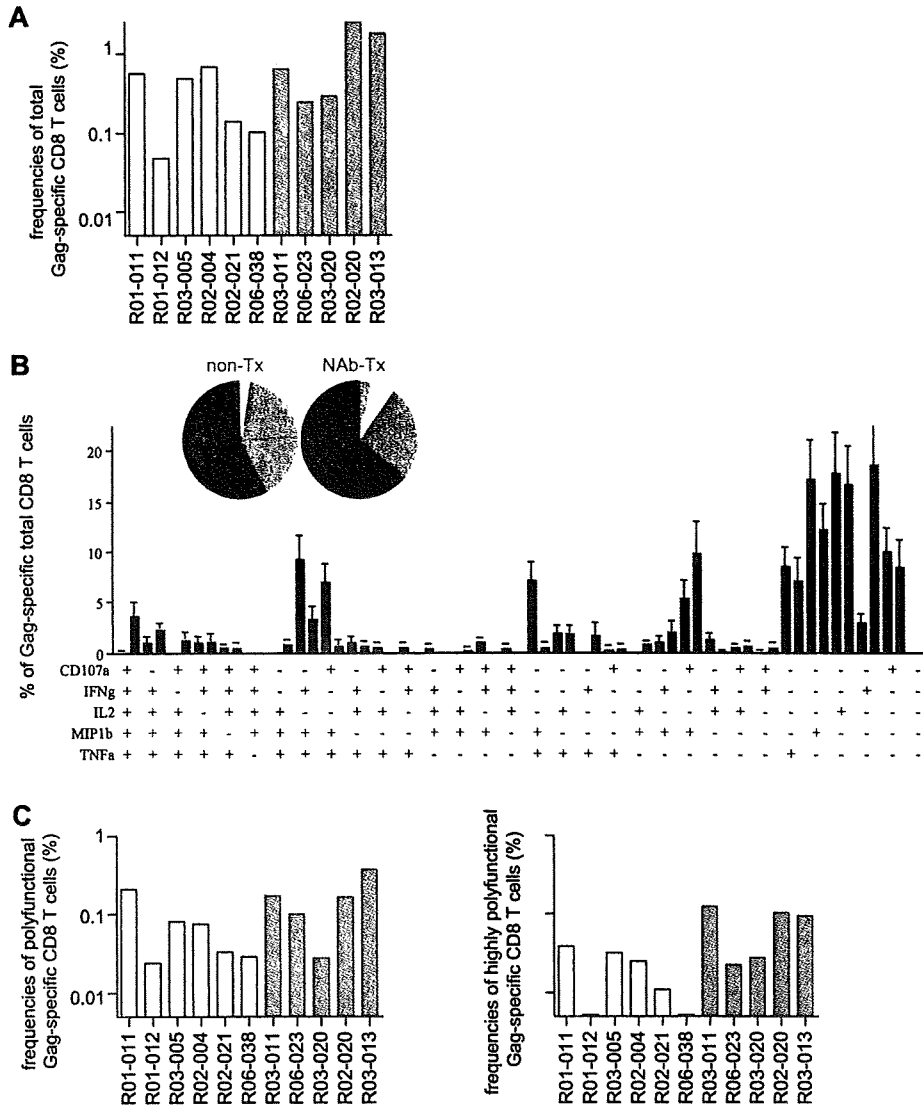


FIG. 8. Gag-specific CD8⁺ T-cell responses in the chronic phase. PBMCs around week 30 postchallenge were stimulated with p55, and specific induction of IFN- γ , TNF- α , IL-2, MIP-1 β , and CD107a in CD8⁺ T cells was examined. (A) Frequencies of total Gag-specific CD8⁺ T cells. (B) Percentages of cells exhibiting Gag-specific induction of single or multiple marker cytokines in total Gag-specific CD8⁺ T cells. See the legend to Fig. 2 for symbols. (C) Frequencies of Gag-specific CD8⁺ T cells exhibiting Gag-specific induction of ≥ 3 marker cytokines (polyfunctional; left) or ≥ 4 marker cytokines (highly polyfunctional; right) in total CD8⁺ T cells. The highly polyfunctional Gag-specific CD8⁺ T-cell frequencies in NAb-immunized macaques were significantly higher than in unimmunized controls ($P = 0.023$).

tent induction of functional virus-specific CD4⁺ T-cell responses in this NAb-triggered SIV control.

All the NAb-immunized macaques had higher frequencies of polyfunctional Gag-specific CD4⁺ T cells than any of the unimmunized controls at week 2, although the two groups possessed similar frequencies of total Gag-specific CD4⁺ T cells. This implies higher polyfunctionality of Gag-specific CD4⁺ T cells in the acute phase in NAb-immunized macaques than in unimmunized controls. HIV-1 is known to preferentially infect HIV-1-specific CD4⁺ T cells (10); virus neutralization may therefore protect virus-specific CD4⁺ T cells from SIV infection. However, it remains unclear whether NABs preferentially protect polyfunctional virus-specific CD4⁺ T

cells. Our previous study suggested augmentation of the Fc-mediated uptake of NAb-virion complexes into dendritic cells following passive NAb immunization (50). This may enhance antigen presentation and induction of polyfunctional virus-specific CD4⁺ T-cell responses in the acute phase. Thus, both NAb-mediated effects, i.e., enhancement of antigen presentation and protection of virus-specific CD4⁺ T cells from viral infection, may contribute to the induction of polyfunctional virus-specific CD4⁺ T cells in the acute phase.

It is thought that potent virus-specific CD4⁺ T-cell responses are important for the control of HIV-1/SIV replication (39). Recent studies analyzing the quality of T-cell responses suggested the possible involvement of polyfunctional CD4⁺

T-cell responses in the control of some viral infections (8, 11, 41). However, there has been no clear evidence indicating association of polyfunctional CD4⁺ T-cell responses with HIV-1/SIV control. These cells are themselves targets for viral infection and killing (10), and most natural HIV-1/SIV infections fail to show efficient induction of potent virus-specific CD4⁺ T-cell responses (52). In the present study, passive NAb immunization of rhesus macaques 1 week after SIV infection resulted in the induction of significantly higher levels of polyfunctional Gag-specific CD4⁺ T-cell responses in the acute phase, followed by SIV control at the set point in the absence of NAb responses. The polyfunctional Gag-specific CD4⁺ T-cell frequencies at week 2 were inversely correlated with plasma viral loads, not at week 2, but at week 5. These results indicate that NABs may facilitate the development and retention of polyfunctional virus-specific CD4⁺ T-cell responses in the very early phase of HIV-1/SIV infection, contributing to subsequent virus control directly or indirectly. Thus, this is the first report documenting an association between polyfunctional CD4⁺ T-cell responses in the acute phase and subsequent SIV control.

Previous studies of the chronic phase of HIV-1 infections have indicated an association between strong HIV-1-specific proliferative CD4⁺ T-cell responses and HIV-1 control, as well as their impairment in HIV-1 infection with uncontrolled viremia (3, 17, 18, 32, 39). In the present study, compared to total Gag-specific CD4⁺ T-cell frequencies in the acute phase, those in the chronic phase were reduced in unimmunized controls, but NAb-immunized macaques maintained similar frequencies in the chronic phase. This difference may reflect virus control in NAb-immunized macaques and high plasma viremia in unimmunized controls. Our analyses of polyfunctional and proliferative responses suggest that these animals maintained functional Gag-specific CD4⁺ T-cell responses in the chronic phase. This may be due to virus control and, conversely, may contribute to sustained virus control.

It has been indicated that virus-specific CD4⁺ T-cell responses facilitate induction of functional virus-specific CD8⁺ T-cell responses (19, 42, 44). Stimulation with peptides would be optimal for analysis of CD8⁺ T-cell responses, but in this study, our first priority was to analyze CD4⁺ T-cell responses, and cell samples were used for the analysis of responses after stimulation with a recombinant Gag p55 protein. Therefore, we obtained results on polyfunctional CD8⁺ T-cell responses after p55-specific stimulation but did not have enough cell samples for analyzing peptide-specific CD8⁺ T-cell responses in the acute phase. In the acute phase, no significant enhancement of polyfunctional Gag-specific CD8⁺ T-cell responses was detected after passive NAb immunization, but this does not exclude the possibility of functional CD8⁺ T-cell induction in NAb-immunized animals, which may be detected by optimal analysis. Indeed, the viral suppression assay showed that CD8⁺ cells able to efficiently suppress SIV replication *in vitro* were induced in the acute phase in those NAb-immunized macaques that contained SIV replication *in vivo*. These highly effective anti-SIV CD8⁺ cell responses, which may be affected not only by CD8⁺ T-cell polyfunctionality, but also by several other factors, are thus likely to be involved in NAb-triggered containment of SIV replication. Our analyses in the chronic phase indicated higher frequencies of highly polyfunctional Gag-spe-

cific CD8⁺ T-cell responses in NAb-immunized macaques, consistent with the previously reported observation in HIV-1-infected nonprogressors (4).

Taken together, the present study indicates that passive NAb immunization of rhesus macaques in the acute phase may be able to trigger rapid induction of polyfunctional Gag-specific CD4⁺ T-cell responses, followed by sustained SIV control in the absence of NAb responses in the chronic phase. These results highlight the importance of the synergy between NAB and T-cell responses in primary virus control, implying that the absence of potent NAB responses in the acute phase of HIV-1/SIV infection may be responsible for failure to control persistent viral replication.

Finally, induction of potent NAB responses is believed to be a promising strategy for AIDS vaccine development. While prechallenge passive NAB immunization studies have previously indicated the possibility of sterile protection against immunodeficiency virus infection in macaques, several studies have suggested difficulty in inducing high levels of NAB responses that are sufficient for sterile protection (16, 28, 35, 43, 48). Our results imply that prophylactic vaccination that elicits NAB responses, even if it does not achieve sterile protection, may contribute to HIV-1/SIV control by secondary NAB responses facilitating functional T-cell induction after viral exposure. Thus, this study indicates a potential for HIV-1/SIV control by synergy between NAB and T-cell responses, providing insights into the development of a prophylactic AIDS vaccine.

ACKNOWLEDGMENTS

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

The animal experiments were conducted through the Cooperative Research Program in Tsukuba Primate Research Center, National Institute of Biomedical Innovation, with the help of the Corporation for Production and Research of Laboratory Primates. We thank H. Akari, F. Ono, K. Komatsuzaki, A. Hiyaoka, H. Ogawa, K. Oto, K. Ishikawa, T. Nakasone, and H. Igarashi for assistance with the animal experiments and M. Miyazawa, M. Yasunami, T. Naruse, and A. Kimura for determining MHC-I haplotypes. We also thank M. Roederer for providing the PESTLE and SPICE software and V. Appay for his technical support and helpful suggestions on analysis of Gag-specific T-cell responses.

REFERENCES

- Almeida, J. R., D. A. Price, L. Papagno, Z. A. Arkoub, D. Sauce, E. Bornstein, T. E. Asher, A. Samri, A. Schnuriger, I. Theodorou, D. Costagliola, C. Rouzioux, H. Agut, A. G. Marcelin, D. Douek, B. Autran, and V. Appay. 2007. Superior control of HIV-1 replication by CD8⁺ T cells is reflected by their avidity, polyfunctionality, and clonal turnover. *J. Exp. Med.* 204:2473–2485.
- Arguello, J. R., A. M. Little, E. Bohan, J. M. Goldman, S. G. Marsh, and J. A. Madrigal. 1998. High resolution HLA class I typing by reference strand mediated conformation analysis (RSCA). *Tissue Antigens* 52:57–66.
- Autran, B., G. Carcelain, T. S. Li, C. Blanc, D. Mathez, R. Tublana, C. Katlama, P. Debre, and J. Leibowitch. 1997. Positive effects of combined antiretroviral therapy on CD4⁺ T cell homeostasis and function in advanced HIV disease. *Science* 277:112–116.
- Betts, M. R., M. C. Nason, S. M. West, S. C. De Rosa, S. A. Migueles, J. Abraham, M. M. Lederman, J. M. Benito, P. A. Goepfert, M. Connors, M. Roederer, and R. A. Koup. 2006. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8⁺ T cells. *Blood* 107:4781–4789.
- Borrow, P., H. Lewicki, B. H. Hahn, G. M. Shaw, and M. B. Oldstone. 1994. Virus-specific CD8⁺ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J. Virol.* 68:6103–6110.
- Brander, C., and B. D. Walker. 1999. T lymphocyte responses in HIV-1

- infection: implication for vaccine development. *Curr. Opin. Immunol.* **11**: 451–459.
7. Burton, D. R., R. C. Desrosiers, R. W. Doms, W. C. Koff, P. D. Kwong, J. P. Moore, G. J. Nabel, J. Sodroski, I. A. Wilson, and R. T. Wyatt. 2004. HIV vaccine design and the neutralizing antibody problem. *Nat. Immunol.* **5**:233–236.
 8. Casazza, J. P., M. R. Betts, D. A. Price, M. L. Precopio, L. E. Ruff, J. M. Brenchley, B. J. Hill, M. Roederer, D. C. Douek, and R. A. Koup. 2006. Acquisition of direct antiviral effector functions by CMV-specific CD4⁺ T lymphocytes with cellular maturation. *J. Exp. Med.* **203**:2865–2877.
 9. Combadiere, B., A. Boissonnas, G. Carcelain, E. Lefranc, A. Samri, F. Bricaire, P. Debre, and B. Antran. 2004. Distinct time effects of vaccination on long-term proliferative and IFN- γ -producing T cell memory to smallpox in humans. *J. Exp. Med.* **199**:1585–1593.
 10. Douek, D. C., J. M. Brenchley, M. R. Betts, D. R. Ambrozak, B. J. Hill, Y. Okamoto, J. P. Casazza, J. Kuruppu, K. Kuustman, S. Wollusky, Z. Grossman, M. Dybul, A. Oxenius, D. A. Price, M. Connors, and R. A. Koup. 2002. HIV preferentially infects HIV-specific CD4⁺ T cells. *Nature* **417**:95–98.
 11. Duvall, M. G., M. L. Precopio, D. A. Ambrozak, A. Jaye, A. J. McMichael, H. C. Whittle, M. Roederer, S. L. Rowland-Jones, and R. A. Koup. 2008. Polyfunctional T cell responses are a hallmark of HIV-2 infection. *Eur. J. Immunol.* **38**:350–363.
 12. Gauduin, M. C., A. Kaur, S. Ahmad, T. Yllma, J. D. Lifson, and R. P. Johnson. 2004. Optimization of intracellular cytokine staining for the quantitation of antigen-specific CD4⁺ T cell responses in rhesus macaques. *J. Immunol. Methods* **288**:61–79.
 13. Gauduin, M. C., R. L. Glickman, R. Meaus, and R. P. Johnson. 1998. Inhibition of simian immunodeficiency virus (SIV) replication by CD8⁺ T lymphocytes from macaques immunized with live attenuated SIV. *J. Virol.* **72**:6315–6324.
 14. Gauduin, M. C., Y. Yu, A. Barabasz, A. Carville, M. Platak, J. D. Lifson, R. C. Desrosiers, and R. P. Johnson. 2006. Induction of a virus-specific effector-memory CD4⁺ T cell response by attenuated SIV infection. *J. Exp. Med.* **203**:2661–2672.
 15. Goulder, P. J., and D. I. Watkins. 2004. HIV and SIV CTL escape: implications for vaccine design. *Nat. Rev. Immunol.* **4**:630–640.
 16. Haigwood, N. L., A. Watson, W. F. Sutton, J. McClure, A. Lewis, J. Ranchalis, B. Travis, G. Voss, N. L. Letvin, S. L. Hu, V. M. Hirsch, and P. R. Johnson. 1996. Passive immune globulin therapy in the SIV/macaque model: early intervention can alter disease profile. *Immunol. Lett.* **51**:107–114.
 17. Harari, A., S. Petitpierre, F. Valléau, and G. Pantaleo. 2004. Skewed representation of functionally distinct populations of virus-specific CD4 T cells in HIV-1-infected subjects with progressive disease: changes after antiretroviral therapy. *Blood* **103**:966–972.
 18. Iyasere, C., J. C. Tilton, A. J. Johnson, S. Younes, B. Yassine-Dlab, R. P. Sekaly, W. W. Kwok, S. A. Migueles, A. C. Laborico, W. L. Shupert, C. W. Hallahan, R. T. Davey, Jr., R. P. Johnson, S. Vogel, J. Metcalf, and M. Connors. 2003. Diminished proliferation of human immunodeficiency virus-specific CD4⁺ T cells is associated with diminished interleukin-2 (IL-2) production and is recovered by exogenous IL-2. *J. Virol.* **77**:10900–10909.
 19. Janssen, E. M., E. E. Lemmens, T. Wolfe, U. Christen, M. G. von Herrath, and S. P. Schoenberg. 2003. CD4⁺ T cells are required for secondary expansion and memory in CD8⁺ T lymphocytes. *Nature* **421**:852–856.
 20. Jin, X., D. E. Bauer, S. E. Tuttleton, S. Lewin, A. Gettler, J. Blanchard, C. E. Irwin, J. T. Safritz, J. Mittler, L. Weinberger, L. G. Kostrikis, L. Zhang, A. S. Perelson, and D. D. Ho. 1999. Dramatic rise in plasma viremia after CD8⁺ T cell depletion in simian immunodeficiency virus-infected macaques. *J. Exp. Med.* **189**:991–998.
 21. Kawada, M., T. Tsukamoto, H. Yamamoto, N. Iwamoto, K. Kurihara, A. Takeda, C. Moriya, H. Takeuchi, H. Akari, and T. Matano. 2008. Gag-specific cytotoxic T lymphocyte-based control of primary simian immunodeficiency virus replication in a vaccine trial. *J. Virol.* **82**:10199–10206.
 22. Kestler, H. W., III, D. J. Ringler, K. Mori, D. L. Panicali, P. K. Sehgal, M. D. Daniel, and R. C. Desrosiers. 1991. Importance of the *nef* gene for maintenance of high virus loads and for development of AIDS. *Cell* **65**:651–662.
 23. Koup, R. A., J. T. Safritz, Y. Cao, C. A. Andrews, G. McLeod, W. Borkowsky, C. Farthing, and D. D. Ho. 1994. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J. Virol.* **68**:4650–4655.
 24. Kwong, P. D., M. L. Doyle, D. J. Casper, C. Cicala, S. A. Leavitt, S. Majeed, T. D. Steenbeke, M. Ventura, I. Chaiken, M. Fung, H. Katinger, P. W. Parren, J. Robinson, D. Van Ryk, L. Wang, D. R. Burton, E. Freire, R. Wyatt, J. Sodroski, W. A. Hendrickson, and J. Arthos. 2002. HIV-1 evades antibody-mediated neutralization through conformational masking of receptor-binding sites. *Nature* **420**:678–682.
 25. Lamoreaux, L., M. Roederer, and R. Koup. 2006. Intracellular cytokine optimization and standard operating procedure. *Nat. Protoc.* **1**:1507–1516.
 26. Li, Q., L. Duan, J. D. Estes, Z. M. Ma, T. Rourke, Y. Wang, C. Rellly, J. Carlis, C. J. Miller, and A. T. Haase. 2005. Peak SIV replication in resting memory CD4⁺ T cells depletes gut lamina propria CD4⁺ T cells. *Nature* **434**:1148–1152.
 27. Loffredo, J. T., E. G. Rakasz, J. P. Giraldo, S. P. Spencer, K. K. Grafton, S. R. Martin, G. Napoe, L. J. Yant, N. A. Wilson, and D. I. Watkins. 2005. Tat_{28–37}SL8-specific CD8⁺ T lymphocytes are more effective than Gag_{181–180}CM9-specific CD8⁺ T lymphocytes at suppressing simian immunodeficiency virus replication in a functional in vitro assay. *J. Virol.* **79**:14986–14991.
 28. Mascola, J. R., G. Stiegler, T. C. VanCott, H. Katinger, C. B. Carpenter, C. E. Hanson, H. Beary, D. Hayes, S. S. Frankel, D. L. Birx, and M. G. Lewis. 2000. Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. *Nat. Med.* **6**:207–210.
 29. Matano, T., M. Kobayashi, H. Igarashi, A. Takeda, H. Nakamura, M. Kano, C. Sugimoto, K. Mori, A. Iida, T. Hirata, M. Hasegawa, T. Yuasa, M. Miyazawa, Y. Takahashi, M. Yasunami, A. Kimura, D. H. O'Connor, D. I. Watkins, and Y. Nagai. 2004. Cytotoxic T lymphocyte-based control of simian immunodeficiency virus replication in a preclinical AIDS vaccine trial. *J. Exp. Med.* **199**:1709–1718.
 30. Matano, T., R. Shibata, C. Silemon, M. Connors, H. C. Laue, and M. A. Martin. 1998. Administration of an anti-CD8 monoclonal antibody interferes with the clearance of chimeric simian/human immunodeficiency virus during primary infections of rhesus macaques. *J. Virol.* **72**:164–169.
 31. Mattapallil, J. J., D. C. Douek, B. Hill, Y. Nishimura, M. A. Martin, and M. Roederer. 2005. Massive infection and loss of memory CD4⁺ T cells in multiple tissues during acute SIV infection. *Nature* **434**:1093–1097.
 32. McNeil, A. C., W. L. Shupert, C. A. Iyasere, C. W. Hallahan, J. A. Mican, R. T. Davey, Jr., and M. Connors. 2001. High-level HIV-1 viremia suppresses viral antigen-specific CD4⁺ T cell proliferation. *Proc. Natl. Acad. Sci. USA* **98**:13878–13883.
 33. National Institute of Infectious Diseases. 2007. Guides for animal experiments performed at National Institute of Infectious Diseases. National Institute of Infectious Diseases, Tokyo, Japan. (In Japanese.)
 34. Nishimura, Y., T. Igarashi, N. L. Haigwood, R. Sadjadpour, O. K. Donau, C. Buckler, R. J. Plishka, A. Buckler-White, and M. A. Martin. 2003. Transfer of neutralizing IgG to macaques 6 h but not 24 h after SHIV infection confers sterilizing protection: implications for HIV-1 vaccine development. *Proc. Natl. Acad. Sci. USA* **100**:15131–15136.
 35. Parren, P. W., P. A. Marx, A. J. Hessel, A. Luckay, J. Harouse, C. Cheng-Mayer, J. P. Moore, and D. R. Burton. 2001. Antibody protects macaques against vaginal challenge with a pathogenic R5 simian/human immunodeficiency virus at serum levels giving complete neutralization in vitro. *J. Virol.* **75**:8340–8347.
 36. Pitcher, C. J., S. I. Hagen, J. M. Walker, R. Lum, B. L. Mitchell, V. C. Maino, M. K. Axthelm, and L. J. Picker. 2004. Development and homeostasis of T cell memory in rhesus macaques. *J. Immunol.* **168**:29–43.
 37. Poignard, P., R. Sabbe, G. R. Picchio, M. Wang, R. J. Gulizia, H. Katinger, P. W. Parren, D. E. Mosler, and D. R. Burton. 1999. Neutralizing antibodies have limited effects on the control of established HIV-1 infection in vivo. *Immunity* **10**:431–438.
 38. Richman, D. D., T. Wrin, S. J. Little, and C. J. Petropoulos. 2003. Rapid evolution of the neutralizing antibody response to HIV type 1 infection. *Proc. Natl. Acad. Sci. USA* **100**:4144–4149.
 39. Rosenberg, E. S., J. M. Billingsley, A. M. Callendo, S. L. Boswell, P. E. Sax, S. A. Kalams, and B. D. Walker. 1997. Vigorous HIV-1-specific CD4⁺ T cell responses associated with control of viremia. *Science* **278**:1447–1450.
 40. Schmitz, J. E., M. J. Kuroda, S. Santra, V. G. Sasseville, M. A. Simon, M. A. Lifton, P. Racz, K. Tenner-Racz, M. Dalesandro, B. J. Scallon, J. Ghayeb, M. A. Forman, D. C. Montefiori, E. P. Rieher, N. L. Letvin, and K. A. Reimann. 1999. Control of viremia in simian immunodeficiency virus infection by CD8⁺ lymphocytes. *Science* **283**:857–860.
 41. Seder, R. A., P. A. Darrach, and M. Roederer. 2008. T-cell quality in memory and protection: implications for vaccine design. *Nat. Rev. Immunol.* **8**:247–258.
 42. Shedlock, D. J., and H. Shen. 2003. Requirement for CD4 T cell help in generating functional CD8 T cell memory. *Science* **300**:337–339.
 43. Shibata, R., T. Igarashi, N. Haigwood, A. Buckler-White, R. Ogert, W. Ross, R. Willey, M. W. Cho, and M. A. Martin. 1999. Neutralizing antibody directed against the HIV-1 envelope glycoprotein can completely block HIV-1/SIV chimeric virus infections of macaque monkeys. *Nat. Med.* **5**:204–210.
 44. Sun, J. C., and M. J. Bevan. 2003. Defective CD8 T cell memory following acute infection without CD4 T cell help. *Science* **300**:339–342.
 45. Trkola, A., H. Kuster, P. Rusert, B. Joos, M. Fischer, C. Leemann, A. Manrique, M. Huber, M. Rehr, A. Oxenius, R. Weber, G. Stiegler, B. Vcelar, H. Katinger, L. Aceto, and H. F. Günthard. 2005. Delay of HIV-1 rebound after cessation of antiretroviral therapy through passive transfer of human neutralizing antibodies. *Nat. Med.* **11**:615–622.
 46. Tsukamoto, T., M. Yuasa, H. Yamamoto, M. Kawada, A. Takeda, H. Igarashi, and T. Matano. 2007. Induction of CD8⁺ cells able to suppress CCR5-tropic simian immunodeficiency virus SIVmac239 replication by controlled infection of CXCR4-tropic simian-human immunodeficiency virus in vaccinated rhesus macaques. *J. Virol.* **81**:11640–11649.
 47. Veazey, R. S., I. C. Tham, K. G. Mansfield, M. DeMarla, A. E. Forand, D. E.

- Shvets, L. V. Chalifoux, P. K. Sehgal, and A. A. Lackner. 2000. Identifying the target cell in primary simian immunodeficiency virus (SIV) infection: highly activated memory CD4⁺ T cells are rapidly eliminated in early SIV infection in vivo. *J. Virol.* 74:57–64.
48. Veazey, R. S., R. J. Shattock, M. Pope, J. C. Kirijan, J. Jones, Q. Hu, T. Ketas, P. A. Marx, P. J. Klasse, D. R. Burton, and J. P. Moore. 2003. Prevention of virus transmission to macaque monkeys by a vaginally applied monoclonal antibody to HIV-1 gp120. *Nat. Med.* 9:343–346.
49. Wei, X., J. M. Decker, S. Wang, H. Hut, J. C. Kappes, X. Wu, J. F. Salazar-Gonzalez, M. G. Salazar, J. M. Kilby, M. S. Saag, N. L. Komarova, M. A. Nowak, B. H. Hahn, P. D. Kwong, and G. M. Shaw. 2003. Antibody neutralization and escape by HIV-1. *Nature* 422:307–312.
50. Yamamoto, H., M. Kawada, A. Takeda, H. Igarashi, and T. Matano. 2007. Post-infection immunodeficiency virus control by neutralizing antibodies. *PLoS ONE* 2:e540.
51. Yang, O. O., S. A. Kalams, A. Trocha, H. Cao, A. Luster, R. P. Johnson, and B. D. Walker. 1997. Suppression of human immunodeficiency virus type 1 replication by CD8⁺ cells: evidence for HLA class I-restricted triggering of cytolytic and noncytolytic mechanisms. *J. Virol.* 71:3120–3128.
52. Zaunders, J. J., M. L. Munier, D. E. Kaufmann, S. Ip, P. Grey, D. Smith, T. Ramacciotti, D. Quan, R. Finlayson, J. Kaldor, E. S. Rosenberg, B. D. Walker, D. A. Cooper, and A. D. Kelleher. 2005. Early proliferation of CCR5⁺ CD38^{low} antigen-specific CD4⁺ Th1 effector cells during primary HIV-1 infection. *Blood* 106:1660–1667.

Impact of Cytotoxic-T-Lymphocyte Memory Induction without Virus-Specific CD4⁺ T-Cell Help on Control of a Simian Immunodeficiency Virus Challenge in Rhesus Macaques[∇]

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Received 2 June 2009/Accepted 29 June 2009

Despite many efforts to develop AIDS vaccines eliciting virus-specific T-cell responses, whether induction of these memory T cells by vaccination before human immunodeficiency virus (HIV) exposure can actually contribute to effective T-cell responses postinfection remains unclear. In particular, induction of HIV-specific memory CD4⁺ T cells may increase the target cell pool for HIV infection because the virus preferentially infects HIV-specific CD4⁺ T cells. However, virus-specific CD4⁺ helper T-cell responses are thought to be important for functional CD8⁺ cytotoxic-T-lymphocyte (CTL) induction in HIV infection, and it has remained unknown whether HIV-specific memory CD8⁺ T cells induced by vaccination without HIV-specific CD4⁺ T-cell help can exert effective responses after virus exposure. Here we show the impact of CD8⁺ T-cell memory induction without virus-specific CD4⁺ T-cell help on the control of a simian immunodeficiency virus (SIV) challenge in rhesus macaques. We developed a prophylactic vaccine by using a Sendai virus (SeV) vector expressing a single SIV Gag₂₄₁₋₂₄₉ CTL epitope fused with enhanced green fluorescent protein (EGFP). Vaccination resulted in induction of SeV-EGFP-specific CD4⁺ T-cell and Gag₂₄₁₋₂₄₉-specific CD8⁺ T-cell responses. After a SIV challenge, the vaccinees showed dominant Gag₂₄₁₋₂₄₉-specific CD8⁺ T-cell responses with higher effector memory frequencies in the acute phase and exhibited significantly reduced viral loads. These results demonstrate that virus-specific memory CD8⁺ T cells induced by vaccination without virus-specific CD4⁺ T-cell help could indeed facilitate SIV control after virus exposure, indicating the benefit of prophylactic vaccination eliciting virus-specific CTL memory with non-virus-specific CD4⁺ T-cell responses for HIV control.

Virus-specific T-cell responses are crucial for controlling human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) replication (3, 4, 12, 20, 28, 36, 37). Therefore, a great deal of effort has been exerted to develop AIDS vaccines eliciting virus-specific T-cell responses (23, 27, 30, 47), but whether this approach actually results in HIV control remains unclear (1, 6). It is important to determine which T-cell responses need to be induced by prophylactic vaccination for HIV control after virus exposure.

Because HIV preferentially infects HIV-specific CD4⁺ T cells (5), induction of HIV-specific memory CD4⁺ T cells by vaccination may increase the target cell pool for HIV infection and could enhance viral replication (42). However, CD4⁺ helper T-cell responses are important for functional CD8⁺ cytotoxic-T-lymphocyte (CTL) induction (11, 40, 43, 46), and it has remained unknown whether HIV-specific memory CD8⁺ T cells induced by vaccination with non-virus-specific CD4⁺ T-cell help (but without HIV-specific CD4⁺ T-cell help) can exert effective responses after virus exposure. Indeed, the real

impact of prophylactic induction of CTL memory itself on HIV replication has not been well documented thus far.

We previously developed a prophylactic AIDS vaccine consisting of DNA priming followed by boosting with a recombinant Sendai virus (SeV) vector expressing SIVmac239 Gag (26). Evaluation of this vaccine's efficacy against a SIVmac239 challenge in Burmese rhesus macaques showed that some vaccinees contained SIV replication whereas unvaccinated animals developed AIDS (15, 27). In particular, vaccination consistently resulted in control of SIV replication in those animals possessing the major histocompatibility complex class I (MHC-I) haplotype *90-120-Ia*. Gag₂₀₆₋₂₁₆ (IINEEAADWDL) and Gag₂₄₁₋₂₄₉ (SSVDEQIQW) epitope-specific CD8⁺ T-cell responses were shown to be involved in SIV control in these vaccinated macaques (14, 16).

In the present study, focusing on CD8⁺ T-cell responses directed against one of these epitopes, we have evaluated the efficacy of a vaccine expressing the Gag₂₄₁₋₂₄₉ epitope fused with enhanced green fluorescent protein (EGFP) against a SIVmac239 challenge in *90-120-Ia*-positive rhesus macaques. The animals exhibited this single-epitope-specific CD8⁺ T-cell response and SeV-EGFP-specific CD4⁺ T-cell responses after vaccination and showed rapid, dominant induction of potent secondary Gag₂₄₁₋₂₄₉-specific CD8⁺ T-cell responses after a SIV challenge. Plasma viral loads in these vaccinees were significantly reduced compared to those of naive controls. These results indicate that induction of CD8⁺ T-cell memory without

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[∇] Published ahead of print on 8 July 2009.

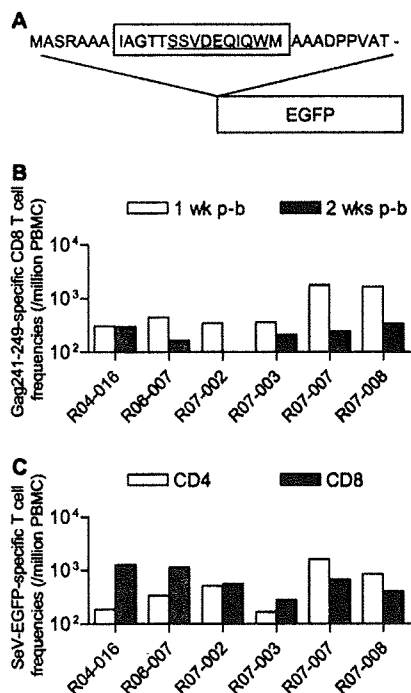


FIG. 1. Gag₂₄₁₋₂₄₉-specific CD8⁺ T-cell induction by prophylactic vaccination. (A) Schema of the cDNA construct encoding the Gag₂₃₆₋₂₅₀-EGFP fusion protein. A DNA fragment that encodes a 31-mer peptide including the Gag₂₃₆₋₂₅₀ sequence was introduced into the 5' end of the EGFP cDNA. (B) Gag₂₄₁₋₂₄₉-specific CD8⁺ T-cell frequencies 1 (open boxes) and 2 weeks (closed boxes) after F(-)SeV-Gag₂₃₆₋₂₅₀-EGFP boosting in group III macaques. (C) SeV-EGFP-specific CD4⁺ (open boxes) or CD8⁺ (closed boxes) T-cell frequencies 2 weeks after F(-)SeV-Gag₂₃₆₋₂₅₀-EGFP boosting in group III macaques. p-b, postboost.

virus-specific CD4⁺ T-cell help by prophylactic vaccination can result in effective CD8⁺ T-cell responses after virus exposure.

MATERIALS AND METHODS

Animal experiments. Burmese rhesus macaques (*Macaca mulatta*) possessing the MHC-I haplotype 90-120-Ia were divided into three groups: unvaccinated group I ($n = 6$), control-vaccinated group II ($n = 6$), and Gag₂₃₆₋₂₅₀-vaccinated group III ($n = 6$). The MHC-I haplotype was determined by reference strand-mediated conformation analysis as described previously (2, 27, 44). Macaque R06-019, administered nonspecific immunoglobulin G 1 week after a SIV challenge, and previously reported macaque R02-007 (15) were included in group I. pGag₂₃₆₋₂₅₀-EGFP-N1 DNA expressing a Gag₂₃₆₋₂₅₀-EGFP fusion protein was constructed from pEGFP-N1 DNA (BD, Tokyo, Japan). The fusion protein was designed to have 31 amino acids including SIVmac239 Gag₂₃₆₋₂₅₀-sequences (IAGTTSSVDEQIQWM) added to the amino-terminal portion of EGFP (Fig. 1A). The group III macaques received 5 mg of pGag₂₃₆₋₂₅₀-EGFP-N1 DNA intramuscularly and 6 weeks later received a single intranasal boost with 6×10^9 cell infectious units of F deletion-containing, replication-defective SeV (24) expressing the Gag₂₃₆₋₂₅₀-EGFP fusion protein (F[-]SeV-Gag₂₃₆₋₂₅₀-EGFP). The group II macaques were primed with pEGFP-N1 DNA and boosted with F(-)SeV-EGFP instead. Approximately 3 months after the boost, these animals and the unvaccinated group I animals were challenged intravenously with 1,000 50% tissue culture infective doses of SIVmac239 (17). All animals were maintained in accordance with the guidelines for animal experiments performed at the National Institute of Infectious Diseases (32).

Analysis of virus-specific CD8⁺ T-cell responses. We measured virus-specific CD8⁺ T-cell levels by flow cytometric analysis of gamma interferon (IFN- γ) induction after specific stimulation as described previously (15, 27). In brief, peripheral blood mononuclear cells (PBMCs) were cocultured with autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines pulsed with

1 μ M SIVmac239 Gag₂₄₁₋₂₄₉ or Gag₂₀₆₋₂₁₆ peptides for Gag₂₄₁₋₂₄₉-specific or Gag₂₀₆₋₂₁₆-specific stimulation. Alternatively, PBMCs were cocultured with B-lymphoblastoid cell lines infected with vesicular stomatitis virus G protein-pseudotyped SIVGPI for SIV-specific stimulation. The pseudotyped virus was obtained by cotransfection of COS-1 cells with a vesicular stomatitis virus G protein expression plasmid and *env* and *nef* deletion-containing simian-human immunodeficiency virus molecular clone (SIVGPI) DNA (26, 41). Intracellular IFN- γ staining was performed with a Cytofix/Cytoperm kit (BD) and fluorescein isothiocyanate-conjugated anti-human CD4, peridinin chlorophyll protein-conjugated anti-human CD8, allophycocyanin (APC)-conjugated anti-human CD3, and phycoerythrin (PE)-conjugated anti-human IFN- γ monoclonal antibodies (BD). Specific T-cell levels were calculated by subtracting nonspecific IFN- γ ⁺ T-cell frequencies from those after peptide-specific or SIV-specific stimulation. Specific T-cell levels lower than 100 per million PBMCs were considered negative.

Analysis of Gag₂₄₁₋₂₄₉-specific cytolytic CD8⁺ T-cell responses. We analyzed Gag₂₄₁₋₂₄₉-specific induction of IFN- γ and CD107a in CD8⁺ T cells. PBMCs were stained with custom-made, PE-conjugated Gag₂₄₁₋₂₄₉ epitope-Mamu-A*90120-5 tetrameric complexes, Gag₂₄₁₋₂₄₉-A*90120-5 tetramers (Medical and Biological Laboratories Co. Ltd., Nagoya, Japan) (45), for 15 min at 37°C and subsequently incubated with anti-human CD107a antibody (BD) for 6 h in the absence or presence of 1 μ M Gag₂₄₁₋₂₄₉ peptide for unstimulated controls or Gag₂₄₁₋₂₄₉-specific stimulation. In both cultures, anti-human CD28 and anti-human CD49d antibodies (5 μ g/ml) (BD) were added for costimulation and monensin (BD) and brefeldin A (Sigma-Aldrich, Tokyo, Japan) were used for inhibition of cytokine secretion. Immunostaining was performed with a Cytofix-Cytoperm kit and the following monoclonal antibodies: fluorescein isothiocyanate-conjugated anti-human perforin (MABTECH), peridinin chlorophyll protein-conjugated anti-human CD4 (BD), APC-conjugated anti-human granzyme B (Invitrogen, Tokyo, Japan), PE-cyanine 7 (PE-Cy7)-conjugated anti-human IFN- γ (BD), APC-Cy7-conjugated anti-human CD3 (BD), energy-coupled-dye-conjugated anti-human CD69 (Beckman Coulter, Tokyo, Japan), Alexa700-conjugated anti-human CD8 (BD), and anti-human CD107a conjugated with Pacific Blue with a Zeon mouse immunoglobulin G1 labeling kit (Invitrogen). Flow cytometric analysis was performed with the FACSAria system (BD). The data were analyzed with FlowJo (version 8.2; TreeStar Inc., Ashland, OR), FACSDiva (BD), PESTLE (version 1.5.4), and SPICE (version 4.1.6) software.

Statistical analysis. Statistical analysis of plasma viral loads in the acute phase (at the peak and week 5) was performed with R version 2.7.1 (R Development Core Team; <http://www.R-project.org/>). Data were log transformed, and a two-tailed one-way analysis of variance, followed by the Shaffer sequentially rejective method of multiple-comparison analysis (39), was performed to estimate differences among groups I, II, and III with overall significance levels set to $\alpha = 0.05$ (two tailed). Statistical analysis of set point plasma viral loads was performed by the nonparametric Kruskal-Wallis test with the sequentially rejective pairwise Mann-Whitney exact test, because we did not assume residual normality and homoscedasticity in set point viral loads, which were mostly below the lower limit of detection in group III animals. Antigen-specific T-cell frequencies were log transformed and compared by unpaired two-tailed t test with significance levels set at $P < 0.05$, and correlation was analyzed by using Prism software version 4.03 (GraphPad Software, Inc., San Diego, CA).

RESULTS

Gag₂₄₁₋₂₄₉-specific CD8⁺ T-cell induction following prophylactic vaccination. Eighteen Burmese rhesus macaques possessing MHC-I haplotype 90-120-Ia were divided into three groups of six animals each (Table 1). Group I received no vaccination, group II received a control vaccine, and group III received a vaccine eliciting Gag₂₄₁₋₂₄₉-specific CD8⁺ T-cell responses. We refer to groups I and II as naive controls in the present study. We constructed a plasmid DNA (pGag₂₃₆₋₂₅₀-EGFP-N1) and an F deletion-containing SeV (F[-]SeV-Gag₂₃₆₋₂₅₀-EGFP) vector both expressing an SIVmac239 Gag₂₃₆₋₂₅₀ (IAGTTSSVDEQIQWM)-EGFP fusion protein to be used for group III vaccination (Fig. 1A). SeV proteins and EGFP have no amino acid sequence identity with SIVmac239. These group III animals received a single intramuscular pGag₂₃₆₋₂₅₀-EGFP DNA injection, followed by a single intra-

TABLE 1. Macaques used in this study

Group	Animal identification codes	Vaccination ^a
I	R02-007, R06-037, R07-001, R07-004, R07-009, R06-019	None
II	R02-008, R05-026, R06-004, R06-014, R06-040, R07-006	Control vaccination [pEGFP-N1 DNA prime, F(-)SeV-EGFP boost]
III	R04-016, R06-007, R07-002, R07-003, R07-007, R07-008	Gag ₂₄₁₋₂₄₉ -specific vaccination [pGag ₂₃₆₋₂₅₀ -EGFP-N1 DNA prime, F(-)SeV-Gag ₂₃₆₋₂₅₀ -EGFP boost]

^a All animals were challenged with SIVmac239.

nasal boost with the F(-)SeV-Gag₂₃₆₋₂₅₀-EGFP vector. Group II animals were administered pEGFP-N1 DNA and the F(-)SeV-EGFP vector, both expressing EGFP instead of Gag₂₃₆₋₂₅₀-EGFP, as a control vaccine.

We measured the antigen-specific CD8⁺ T-cell responses in these macaques 1 or 2 weeks after the SeV boost by detection of specific IFN- γ induction. All group III macaques showed efficient Gag₂₄₁₋₂₄₉-specific CD8⁺ T-cell induction after the F(-)SeV-Gag₂₃₆₋₂₅₀-EGFP boost (Fig. 1B). In these animals, we also confirmed SeV-EGFP-specific CD8⁺ and CD4⁺ T-cell responses (Fig. 1C) but did not detect Gag₂₀₆₋₂₁₆-specific CD8⁺ T-cell responses, which are dominantly induced in 90-120-Ia-positive macaques by Gag-expressing SeV vaccination (14). We have never found Gag₂₃₆₋₂₅₀-specific CD4⁺ T-cell responses in any previously examined animals, and as expected, analyses with the Gag₂₃₆₋₂₅₀ peptide did not detect Gag₂₃₆₋₂₅₀-specific CD4⁺ T-cell responses in any of the group III animals in the present study. In group II animals, we detected SeV-EGFP-specific T-cell responses but not Gag₂₃₆₋₂₅₀-specific T-cell responses after the F(-)SeV-EGFP boost (data not shown).

Control of an SIV challenge in vaccinees. Group I (unvaccinated), II (control-vaccinated), and III (Gag₂₃₆₋₂₅₀-vaccinated) macaques were challenged intravenously with SIVmac239. Plasma viral loads in these animals were examined after the challenge (Fig. 2A). Most of the group I and II animals failed to contain SIV replication, although plasma viremia became undetectable at week 12 in one animal in group I (R06-037) and one in group II (R06-004). No significant differences were observed between groups I and II in plasma viral loads at the peak, at week 5, at week 12, or around week 24 after the challenge. In contrast, most group III animals contained SIV replication; plasma viral loads became undetectable after week 5 in five of the six animals (Fig. 2A). Plasma viral loads in these animals were significantly lower than those in unvaccinated group I and those in control-vaccinated group II at the peak, at week 5, and at the set point (Fig. 2B). Thus, the prophylactic vaccination inducing Gag₂₄₁₋₂₄₉ single-epitope-specific CD8⁺ T-cell responses resulted in a significant reduction of peak and subsequent viral loads after the SIV challenge. No significant difference in peripheral CD4⁺ T-cell counts was observed among these three groups (Fig. 2C).

Dominant Gag₂₄₁₋₂₄₉-specific CD8⁺ T-cell responses in vaccinees after a SIV challenge. We assessed virus-specific CD8⁺ T-cell responses at weeks 2 and 12 after a SIV challenge by

measuring antigen-specific IFN- γ induction. Gag₂₄₁₋₂₄₉-specific CD8⁺ T-cell responses were undetectable or marginal in some naive controls (group I and II) but were efficiently induced in all of the group III animals (Fig. 3A). In most of the naive controls, Gag₂₀₆₋₂₁₆-specific CD8⁺ T-cell responses were induced equivalently or more efficiently than Gag₂₄₁₋₂₄₉-specific CD8⁺ responses, whereas all of the group III animals showed dominant induction of Gag₂₄₁₋₂₄₉-specific CD8⁺ T-cell responses. In these group III animals, Gag₂₀₆₋₂₁₆-specific CD8⁺ T-cell responses were inefficient but frequencies of CD8⁺ T cells exhibiting Gag₂₄₁₋₂₄₉-specific IFN- γ induction were significantly higher than in naive controls at week 2 (Fig. 3B) and week 12. Gag₂₄₁₋₂₄₉-specific CD8⁺ T-cell frequencies at week 2 inversely correlated with peak viral loads (Fig. 3C).

We also tested SIV-specific CD8⁺ T-cell responses in these animals (Fig. 4). We used *env* and *nef* deletion-containing simian-human immunodeficiency virus molecular clone DNA SIVGPI containing the genes encoding SIVmac239 Gag, Pol, Vif, Vpx, and a part of Vpr and measured the frequencies of CD8⁺ T cells responding to SIVGPI-transduced cells (referred to as SIV-specific CD8⁺ T cells) as described previously (15). Naive controls (groups I and II) and vaccinees (group III) were found to possess similar levels of SIV-specific CD8⁺ T cells at week 2 and week 12.

In our previous study (27), all of the 90-120-Ia-positive macaques vaccinated with Gag-expressing SeV contained SIV replication with rapid selection of a *gag* mutation (GagL216S), resulting in escape from Gag₂₀₆₋₂₁₆-specific CD8⁺ T-cell recognition at week 5, implicating Gag₂₀₆₋₂₁₆-specific CD8⁺ T-cell responses (rather than Gag₂₄₁₋₂₄₉-specific CD8⁺ T-cell responses) in viral control. In the present study, however, five of six Gag₂₃₆₋₂₅₀-vaccinated animals controlled SIV replication and had undetectable set point viremia without selection of *gag* mutation over 5 weeks (data not shown). No *gag* mutation was selected at week 5 in naive controls, either. These results indicate that in the group III macaques, dominantly induced Gag₂₄₁₋₂₄₉-specific CD8⁺ T-cell responses in the acute phase play an important role in this vaccine-based SIV control.

Higher Gag₂₄₁₋₂₄₉-specific effector memory CD8⁺ T-cell frequencies in vaccinees. We then examined Gag₂₄₁₋₂₄₉-specific CD8⁺ T-cell frequencies in these macaques by using PE-conjugated Gag₂₄₁₋₂₄₉-A*90120-5 tetramers. In group III animals, Gag₂₄₁₋₂₄₉-specific tetramer⁺ CD8⁺ T cells were still detectable just before the SIV challenge, and their frequencies increased greatly after the challenge; most of the vaccinees exhibited a >10-fold increase at week 2 compared to prechallenge levels (Fig. 5A). Increases in tetramer⁺ CD28⁺ CD8⁺ T-cell frequencies after a challenge were especially marked (>30-fold) (Fig. 5B). Indeed, within the tetramer⁺ cells, the ratio of CD28⁺ cells increased after a challenge and these cells became predominant at week 2. Analysis of an effector memory subset delineated by the CD95⁺ CD28⁺ phenotype (29, 34) revealed significantly higher frequencies of Gag₂₄₁₋₂₄₉-specific tetramer⁺ CD95⁺ CD28⁺ CD8⁺ T cells in group III than in naive controls (Fig. 5C). These results suggest efficient responses of Gag₂₄₁₋₂₄₉-specific CD8⁺ T cells with effector function in the acute phase in group III animals.

Gag₂₄₁₋₂₄₉-specific cytolytic CD8⁺ T-cell responses in vaccinees. To further investigate the cytolytic quality of Gag₂₄₁₋₂₄₉-specific CD8⁺ T-cell responses after a challenge, we examined