図 3　自然免疫系のマスト細胞による IgE 産生制御機構
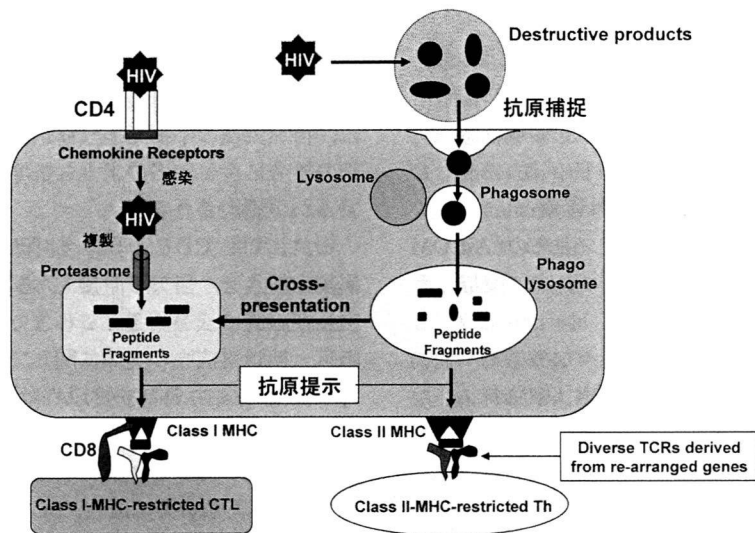

図 4　樹状細胞による Cross-presentation と抗原提示
＊文献 11) より改変

疫担当細胞によるレセプター構築により異物に対する記憶形成が誘導される（図2）。

　こうした例は IgE という特異的な抗体を介したアレルギー病態の場合にも認められる。この IgE という抗体は獲得免疫システムに属する B リンパ球が産生するものであるが，この B 細胞にアレルギーの原因物質（アレルゲン）を提示し特異的な IgE の産生を促すのが，原因物質に対する記憶は有していないものの速やかに侵入アレルゲンをキャッチする，体表面に配置された自然免疫系に属するマスト細胞であることが判明してきた[5]。このマスト細胞は自らの

表面に構築を指示した IgE を提示するためのレセプター，IgE-FcR を発現し，アレルゲンが体内に侵入しようとした場合，それらを特異的に効率良く捕捉し，自らに発現したクラス II MHC 分子を介して Th2 タイプのヘルパー T 細胞を刺激し，IgE 産生の準備状態にある B 細胞に同一の個体産生を命ずる（図 3）。このようにして体内に侵入したアレルゲンに遭遇する度に，アレルゲン特異的な IgE を産生する抗体産生細胞が刺激され，IgE の産生・放出が加速される。それ故，血清中の IgE が高値を示す患者はアレルゲンによる頻回の暴露があったことを物語っている。

このように我々の獲得免疫系は，自然免疫の中心に存在する記憶を持たない樹状細胞あるいは体表面に局在するマスト細胞からの情報提示，特にMHC分子を介した情報提示を介して誘導される。その意味でMHC分子は獲得免疫系への記憶を促すための，自然免疫担当細胞に設置された個体内情報提示分子と考えられる。

2. 自然界に存在する自然免疫系活性化物質と cross-presentation

我々は以前，ウイルスを構成する核酸群を代表するdsRNAとしてのPoly（I：C）がウイルス蛋白を取り込んだ自然免疫系の樹状細胞群に発現したToll-like receptor（TLR）3を刺激し，自らのクラスI MHC分子を介して獲得免疫系のCD8T細胞に記憶すべきウイルス断片を提示し，特異的なレセプター発現を誘導することを報告した[6]。一般に，細胞から取り込まれた蛋白質は断片化されクラスII MHCとともに細胞表面に提示され，CD4分子を発現したヘルパーT細胞を活性化すると考えられていた[7]が，このようにウイルス自体に内在する核酸群の存在によってあたかも細胞内で産生されたウイルス蛋白断片のようにクラスI MHCから提示され，CD8陽性のキラーT細胞を誘導することが判明した。このような現象を樹状細胞内で誘発される「cross-presentation」と呼ぶ。我々は，こうした「cross-presentation」が，抗原蛋白を細菌外毒素としての「コレラ毒素」[8]や，樹皮から抽出したサポニン由来の「ISCOM（immunostimulating complex）」[9]とともに生体に投与した場合にも誘発されることを見いだした。

こうした事実は，自然界に存在する様々な微生物や植物には，自然免疫系，特にその中枢を担う樹状細胞群に作用し捕捉した抗原分子をクラスI MHCから選択的に提示し，感染細胞そのものの制御を担う微生物特異的なCD8陽性キラーT細胞を誘導する機構が内在していることを物語っている（図4）。異物に直接的に遭遇する樹状細胞が有する特殊な機能である「cross-presentation」を介して誘導されたCD8陽性キラーT細胞は，再度の微生物の侵入を侵入細胞上に発現しているクラスI MHC分子を介して効率良くキャッチし，速やかに感染細胞を制御する。ただし，このキラーT細胞に遺伝子再配列（re-arrangement）を介して異物特異的な記憶形成を誘発することができるのは単なる感染細胞ではなく，共刺激分子を発現した樹状細胞群であると考えられる。

3. MHC分子とCD1分子

これまで述べてきたように，樹状細胞の表面には2種類の異なる抗原提示分子が発現している。一つは，蛋白ペプチド抗原を提示し獲得免疫担当T細胞群に働きかけるこ

とによって，特異的なレセプターを細胞内遺伝子群の再構築（re-arrangement）を介して誘導するMHC分子群であり，もう一つは自然免疫担当T細胞群（γδ型T細胞，NKT細胞，そして一部のCD1-拘束性T細胞）に予め発現している固定型レセプター群（invariant chain）に脂質・糖脂質抗原を提示し，それらを活性化するCD1分子群である。

このMHC分子はそれぞれの個体内で同一であるものの，個体が異なれば千差万別（individually-restricted）であることから，個体内における情報伝達分子であると考えられる。従って，MHC分子が異なる細胞の混入は，体内情報伝達障害をもたらすものと想定される。その故に，各個体内では僅かな変異を有した細胞の混入も許容しないようなシステムが構築されており，それが拒絶反応を誘発するものと推測される。これらMHC分子が体内侵入微生物由来のペプチド断片を提示する場合，その提示抗原は抗原を捕捉・分解した抗原提示細胞に由来するため，通常は個々の個体で異なると考えられる。また同一個体内にあっても，存在する抗原提示細胞の状態あるいは種類によって，提示される異物情報であるペプチド断片が全く同一のものであるか否かは今後の検討を待たねばならない。何れにせよ，体内に侵入した微生物に対する獲得免疫発動の鍵を握るのは，自然免疫の中枢を担う樹状細胞群であり，この樹状細胞の指令によってどのような抗原分子に対する記憶を形成するかが規定される。

これに対しCD1分子群は，脂質・糖脂質抗原を提示し異物の侵入を，自分の直接的な配下とも言うべき自然免疫担当細胞群に伝達する。これまでのところ，微生物由来の脂質・糖脂質抗原の実体に関しては，樹状細胞に感染性を有する結核菌由来の物質以外は全く明らかになっておらず，自然免疫担当細胞群がCD1分子群を介して提示されたどのような抗原を認識し活性化するのか，更なる解明を期待したい。筆者等は最近，BCG感染樹状細胞によって活性化されたNKT細胞およびγδT細胞が，膀胱癌の増殖抑制あるいは腫瘍破壊に強く関与することを報告した[10]。こうした事実は，体表面の皮膚組織あるいは粘膜組織に発生した腫瘍細胞の制御に，活性化した自然免疫担当細胞が多大な効果を有することを示しており，体表面から侵入し初めて遭遇したウイルスの制御においても，重要な意義を有することを示唆している。

おわりに：自然免疫活性化を考慮したエイズワクチン開発の重要性

以上より，ウイルスに対するワクチン開発を考える場合，体内獲得免疫を誘発するためにも，また初期侵入時における侵入微生物を制御するためにも，こうした自然免疫応答を増強・活性化することが非常に重要であり，これま

で人類が多くの伝染病を克服してきた経緯を考えた場合,その鍵は侵入微生物そのものに内在していることを念頭に置くべきであろう[11]。この際,腫瘍細胞そのものでは特異的な CD8 陽性キラー T 細胞を主体とした獲得免疫が誘導できなかったように,ウイルス感染細胞そのものでは効率的な獲得免疫応答は誘発できない可能性を想定しワクチンの開発を進めることが肝要である。従って,樹状細胞をも標的とするエイズウイルスに対するワクチンを考えた場合,樹状細胞の機能を保持しつつ,初期侵入ウイルス制御のための CD1 分子を介した自然免疫を活性化し,かつウイルス感染細胞の制御を担う MHC 分子を介した獲得免疫の誘導する必要がある。このような視点から,果たしてこれまでのワクチン候補が自然免疫の活性化を抑制するような作用は無かったか,また真に各個体内で樹状細胞が提示する可能性のあるエピトープペプチドを用いたものであったか,再考する必要があろう。

こうした中,我々は母乳中の樹状細胞前駆細胞群が HIV の感染伝播の鍵を握ること[12],HAART 治療 HIV-1 感染者において末梢血中のウイルスは検出感度以下であっても粘膜樹状細胞中にはウイルス粒子が検出されること(論文投稿中),そして HIV-1 由来の nef 遺伝子産物が樹状細胞表面の MHC 分子のみならず CD1 分子群の発現低下を引き起こし抗原提示能を阻害すること[3]を見いだすとともに,nef 遺伝子の発現を抑制したウイルス粒子を作製し,それらが抗原提示分子の発現抑制をしないことを確認した[13]。またサルモデルにおいて nef 遺伝子を欠損させた SHIV を接種した場合,同一ウイルスの攻撃接種に対し強い抵抗力が誘導されることを観察している[14]。

さらに最近筆者等は,担癌マウスにおいて樹状細胞の機能を選択的に高め,癌細胞に僅かなダメージを与えることによって,効率的な獲得免疫が自然に誘発されることを見いだした(論文 Revised)。このことは,樹状細胞を刺激・活性化した状態に制御可能な微量ウイルス粒子を接種することによって,各個体の中で「cross-presentation」された抗原分子による適切な獲得免疫が誘発されることを示唆している。共通の CD1 分子を有する種が同じ感染症に罹患すること,そしてこれまで多くの感染症を共通の CD1 分子群を有した人類が克服してきた事実は,樹状細胞活性化による CD1 分子群を介した自然免疫の活性化を介した侵入微生物に対するダメージが誘発されれば,おのずと適切な獲得免疫が体内誘発される,といったワクチン開発に対する考え方のパラダイムシフトが必要となってきていることを物語っている。

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

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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 NAbs, 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 NAbs 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 associated 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 NAbs 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 NAbs 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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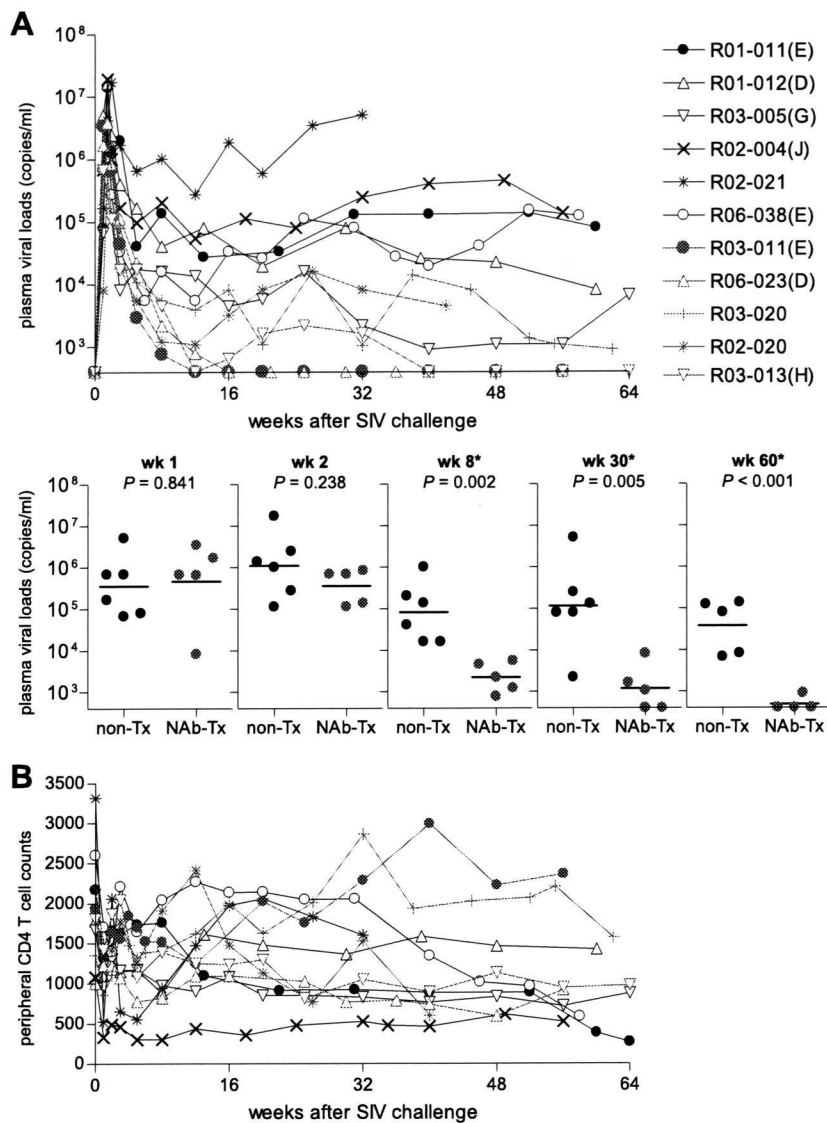▽ Published ahead of print on 18 March 2009.

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 µl) 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-
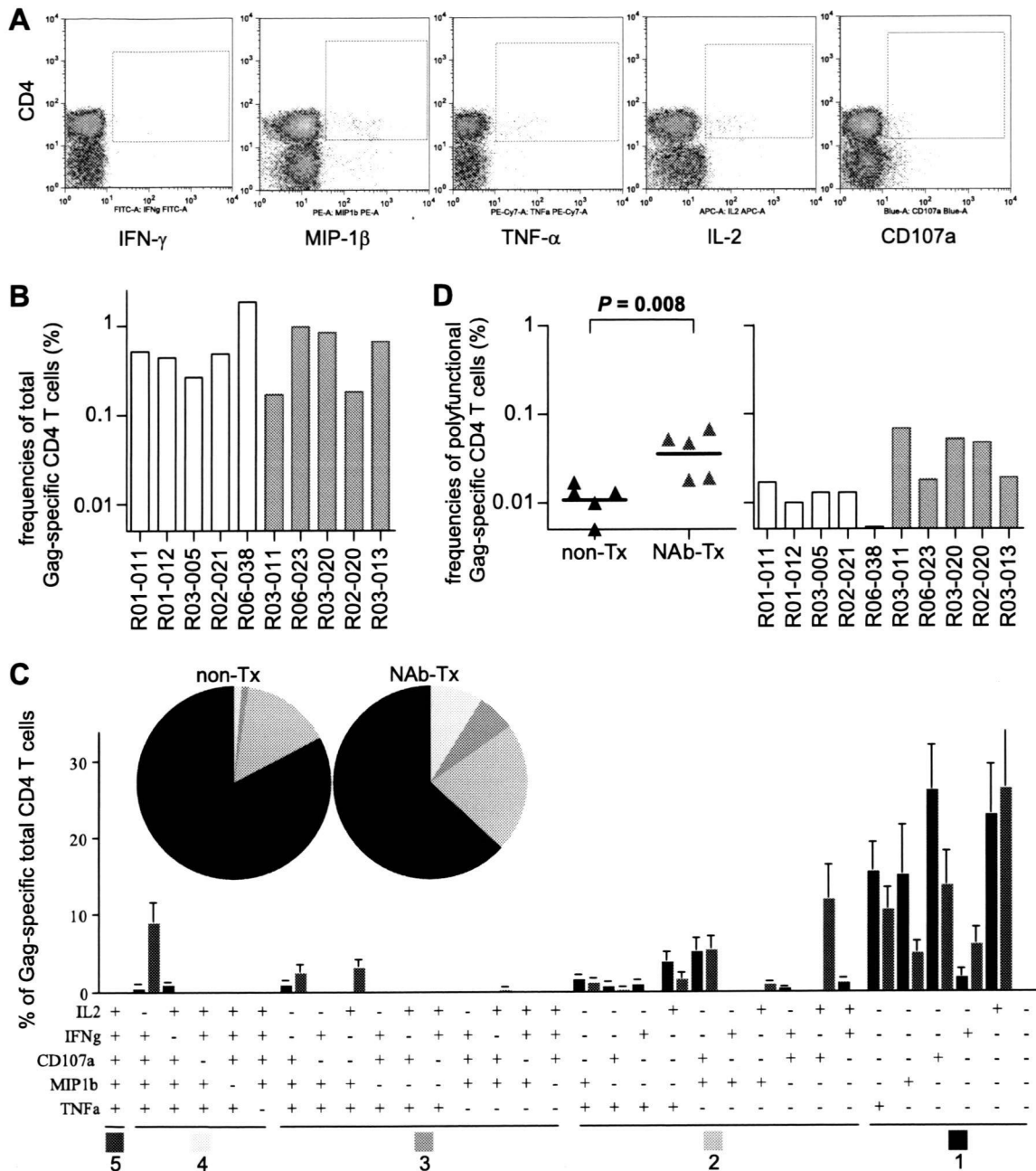
FIG. 2. Gag-specific CD4^{+} T-cell responses in the acute phase. PBMCs at week 2 postchallenge were stimulated with a recombinant SIV Gag p55, and the specific induction of five marker cytokines (IFN-γ, TNF-α, IL-2, MIP-1β, and CD107a) in CD4^{+} T cells was examined. PBMCs from macaque R02-004 were unavailable and therefore could not be included in this analysis. (A) Representative dot plots showing responses of IFN-γ, TNF-α, IL-2, MIP-1β, 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-1β, 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 (TCID$_{50}$) 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 TCID$_{50}$ of SIVmac239 for 45 min (5 μl of diluted sample was incubated with 5 μl of virus in each mixture) and added to 5 × 10^4 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 endpoint. 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 1β (MIP-1β), 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 μg/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 μg/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 CytofixCytoperm kit (BD) and the following monoclonal antibodies: fluorescein isothiocyanate-conjugated anti-human IFN-γ (BD), phycoerythrin (PE)-conjugated anti-human MIP-1β (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-1β, 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 μg/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 μg/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 μg/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.
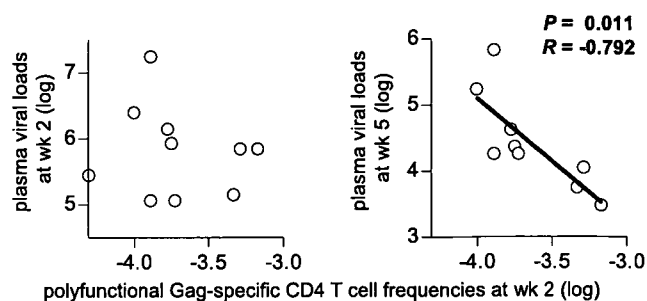

FIG. 3. Analysis of correlation between polyfunctional Gag-specific CD4$^+$ T-cell frequencies (log) at week (wk) 2 and plasma viral loads (log) at week 2 (left) or week 5 (right). Inverse correlation is shown on the right ($P = 0.011$; $R = -0.792$), but not on the left ($P = 0.694$; $R = -0.143$).

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 SIV-mac239 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
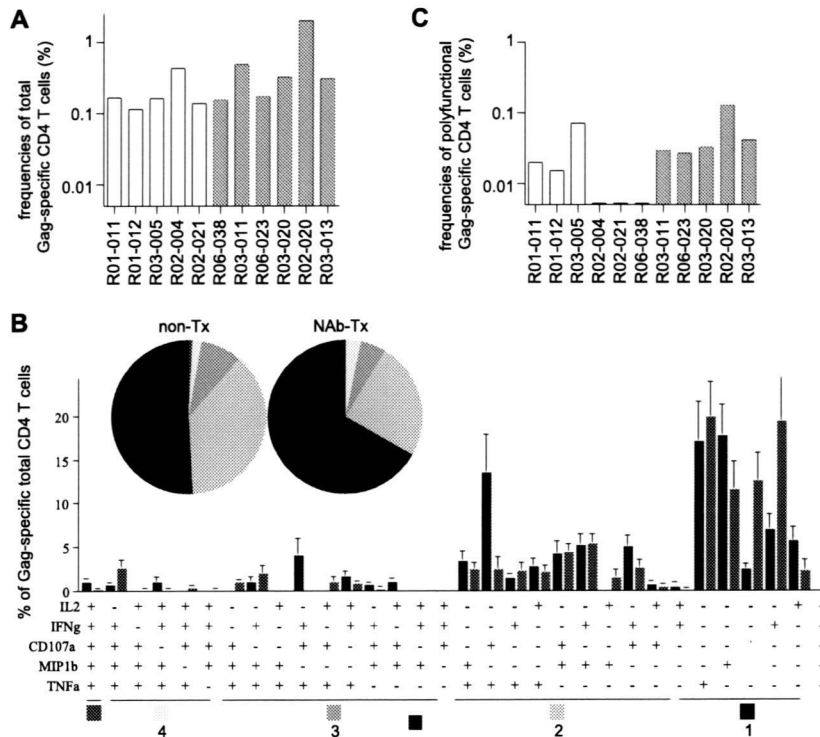
A

C

B

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-1β, 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,
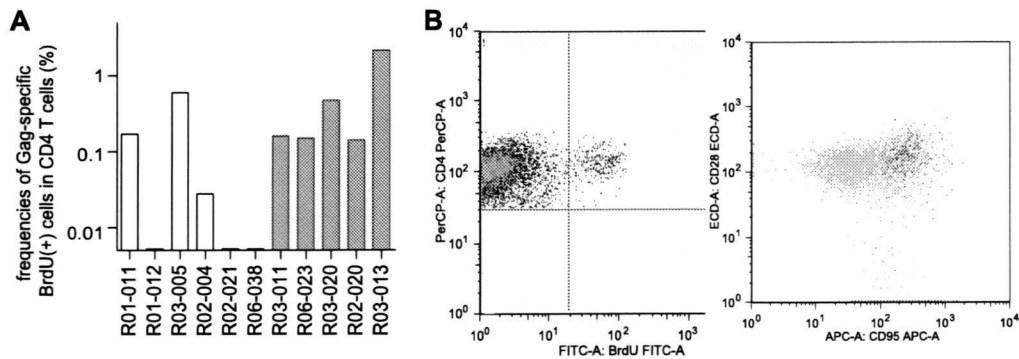
A

B

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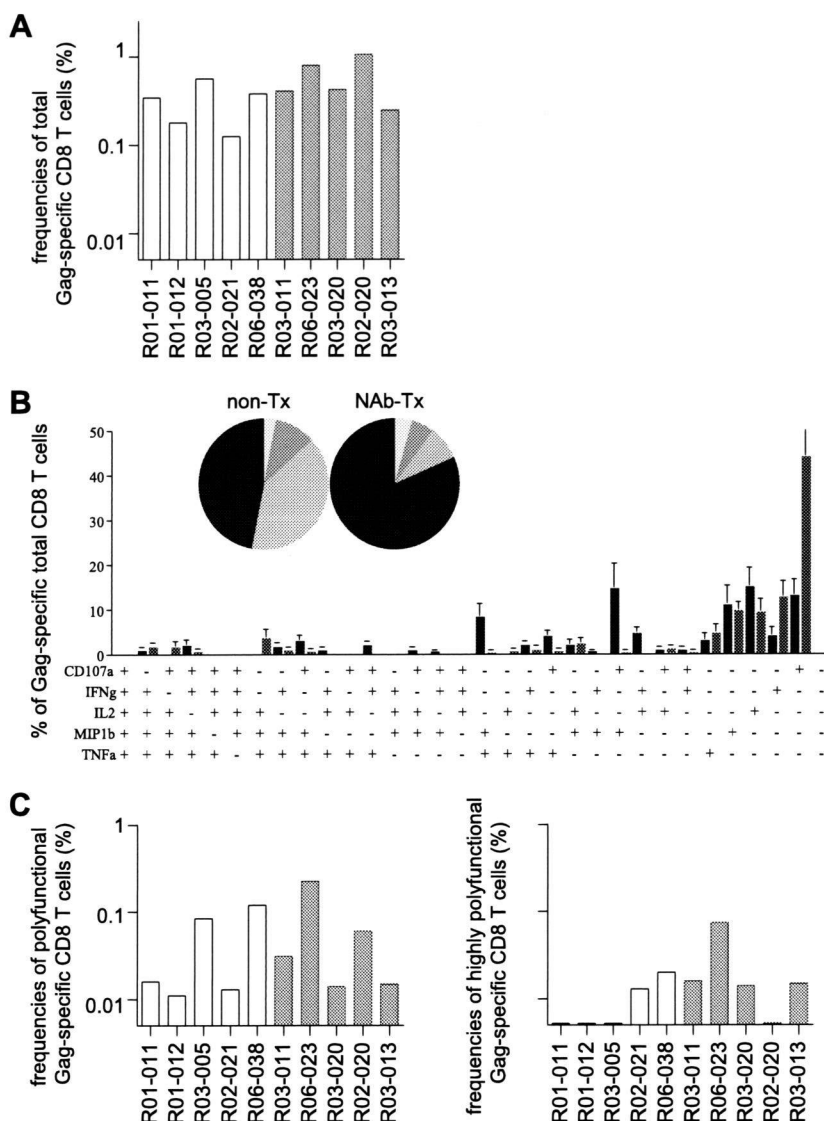
FIG. 6. Gag-specific CD8$^+$ T-cell responses in the acute phase. PBMCs at week 2 were stimulated with p55, and specific induction of IFN-γ, TNF-α, IL-2, MIP-1β, and CD107a in CD8$^+$ T cells was examined. (A) Frequencies of total Gag-specific CD8$^+$ T cells. (B) Percentages of cells exhibiting Gag-specific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

TNF-α, IL-2, and MIP-1β 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-1β, 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-1β, 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 $\geq$3 marker cytokines (Fig. 2C, sum of red, yellow, and green) in the Gag-specific CD4$^+$ 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-
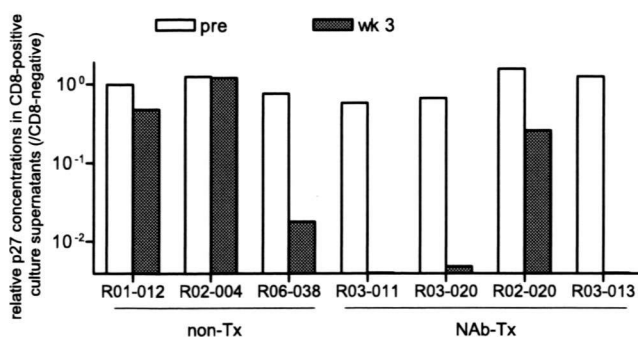

FIG. 7. Anti-SIV efficacy in vitro of CD8^{+} cells. PBMC-derived CD8^{−} (target) cells infected with SIVmac239 were cultured alone (no CD8) or cocultured with autologous PBMC-derived CD8^{+} (effector) cells obtained prechallenge (pre) or at week 3 postchallenge (wk 3) at an E/T ratio of 1:4. The results 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-
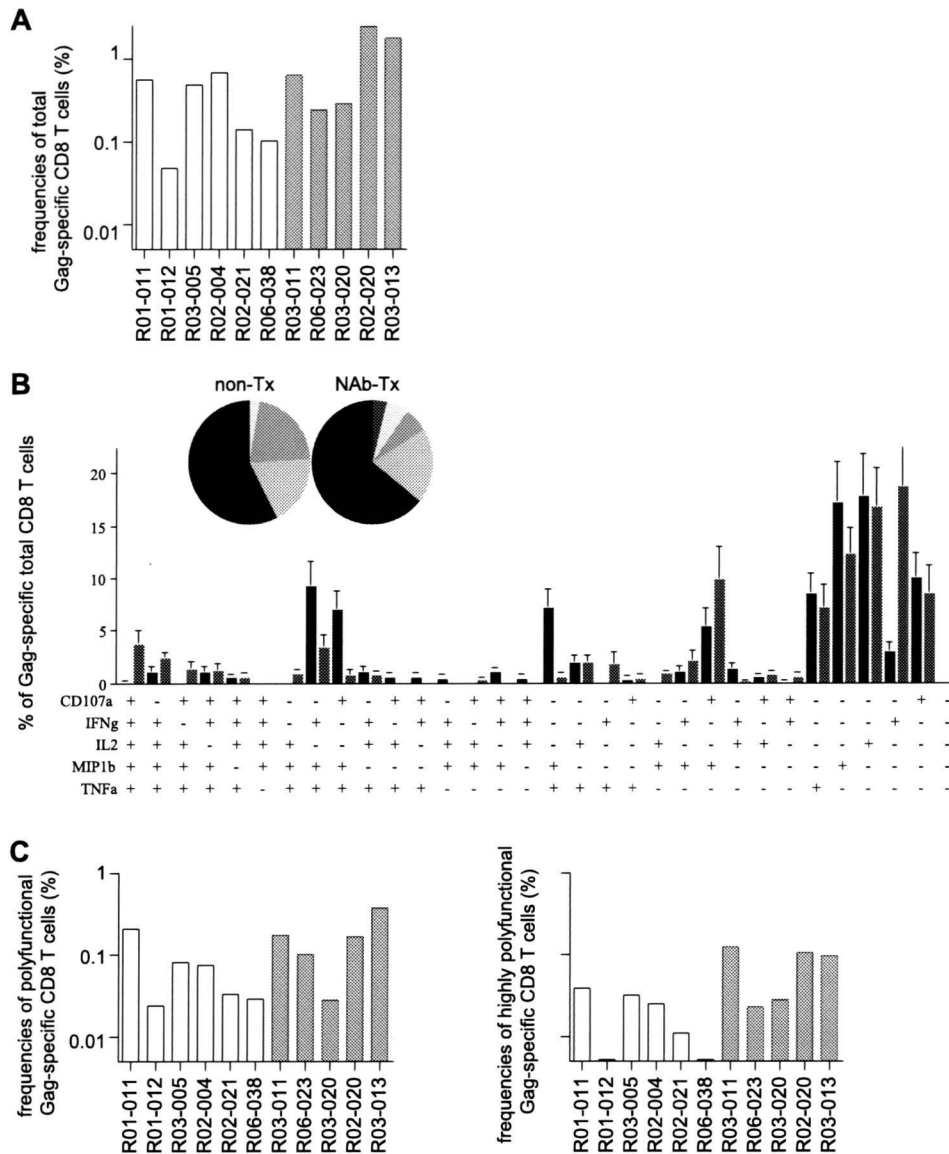
FIG. 8. Gag-specific CD8^{+} T-cell responses in the chronic phase. PBMCs around week 30 postchallenge were stimulated with p55, and specific induction of IFN-γ, TNF-α, IL-2, MIP-1β, and CD107a in CD8^{+} T cells was examined. (A) Frequencies of total Gag-specific CD8^{+} T cells. (B) Percentages of 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.

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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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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 Gag$_{241-249}$ CTL epitope fused with enhanced green fluorescent protein (EGFP). Vaccination resulted in induction of SeV-EGFP-specific CD4⁺ T-cell and Gag$_{241-249}$-specific CD8⁺ T-cell responses. After a SIV challenge, the vaccinees showed dominant Gag$_{241-249}$-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*. Gag$_{206-216}$ (IINEEAADWDL) and Gag$_{241-249}$ (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 Gag$_{241-249}$ 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 Gag$_{241-249}$-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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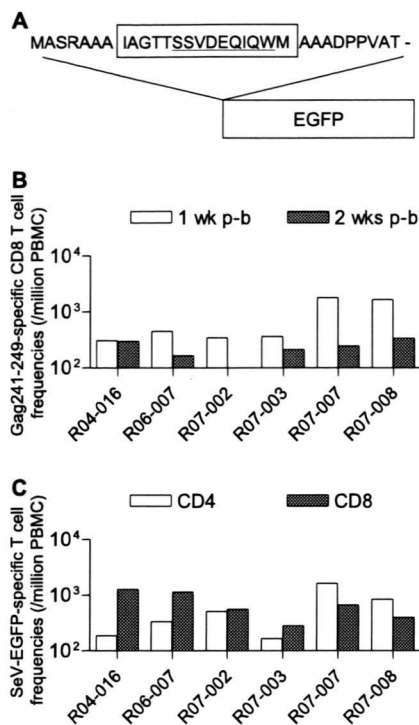9339

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FIG. 1. Gag$_{241-249}$-specific CD8$^+$ T-cell induction by prophylactic vaccination. (A) Schema of the cDNA construct encoding the Gag$_{236-250}$-EGFP fusion protein. A DNA fragment that encodes a 31-mer peptide including the Gag$_{236-250}$ sequence was introduced into the 5′ end of the EGFP cDNA. (B) Gag$_{241-249}$-specific CD8$^+$ T-cell frequencies 1 week (open boxes) and 2 weeks (closed boxes) after F($-$)SeV-Gag$_{236-250}$-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-Gag$_{236-250}$-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 Gag$_{236-250}$-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. pGag$_{236-250}$-EGFP-N1 DNA expressing a Gag$_{236-250}$-EGFP fusion protein was constructed from pEGFP-N1 DNA (BD, Tokyo, Japan). The fusion protein was designed to have 31 amino acids including SIVmac239 Gag$_{236-250}$-sequences (IAGTTSSVDEQIQWM) added to the amino-terminal portion of EGFP (Fig. 1A). The group III macaques received 5 mg of pGag$_{236-250}$-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 Gag$_{236-250}$-EGFP fusion protein (F[$-$]SeV-Gag$_{236-250}$-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 μM SIVmac239 Gag$_{241-249}$ or Gag$_{206-216}$ peptides for Gag$_{241-249}$-specific or Gag$_{206-216}$-specific stimulation. Alternatively, PBMCs were cocultured with B-lymphoblastoid cell lines infected with vesicular stomatitis virus G protein-pseudotyped SIVGP1 for SIV-specific stimulation. The pseudotyped virus was obtained by cotransfection of COS-1 cells with a vesicular stomatitis virus G protein expression plasmid and *env* and *nef* deletion-containing simian-human immunodeficiency virus molecular clone (SIVGP1) DNA (26, 41). Intracellular IFN-γ staining was performed with a CytofixCytoperm 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 Gag$_{241-249}$-specific cytolytic CD8$^+$ T-cell responses. We analyzed Gag$_{241-249}$-specific induction of IFN-γ and CD107a in CD8$^+$ T cells. PBMCs were stained with custom-made, PE-conjugated Gag$_{241-249}$ epitope-Mamu-A*90120-5 tetrameric complexes, Gag$_{241-249}$-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 μM Gag$_{241-249}$ peptide for unstimulated controls or Gag$_{241-249}$-specific stimulation. In both cultures, anti-human CD28 and anti-human CD49d antibodies (5 μg/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 α = 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

Gag$_{241-249}$-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 Gag$_{241-249}$-specific CD8$^+$ T-cell responses. We refer to groups I and II as naive controls in the present study. We constructed a plasmid DNA (pGag$_{236-250}$-EGFP-N1) and an F deletion-containing SeV (F[$-$]SeV-Gag$_{236-250}$-EGFP) vector both expressing an SIVmac239 Gag$_{236-250}$ (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 pGag$_{236-250}$-EGFP DNA injection, followed by a single intra-

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-Gag$_{236-250}$-EGFP vector. Group II animals were administered pEGFP-N1 DNA and the F(−)SeV-EGFP vector, both expressing EGFP instead of Gag$_{236-250}$-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 Gag$_{241-249}$-specific CD8$^+$ T-cell induction after the F(−)SeV-Gag$_{236-250}$-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 Gag$_{206-216}$-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 Gag$_{236-250}$-specific CD4$^+$ T-cell responses in any previously examined animals, and as expected, analyses with the Gag$_{236-250}$ peptide did not detect Gag$_{236-250}$-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 Gag$_{236-250}$-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 (Gag$_{236-250}$-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 Gag$_{241-249}$ 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 Gag$_{241-249}$-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. Gag$_{241-249}$-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, Gag$_{206-216}$-specific CD8$^+$ T-cell responses were induced equivalently or more efficiently than Gag$_{241-249}$-specific CD8$^+$ responses, whereas all of the group III animals showed dominant induction of Gag$_{241-249}$-specific CD8$^+$ T-cell responses. In these group III animals, Gag$_{206-216}$-specific CD8$^+$ T-cell responses were inefficient but frequencies of CD8$^+$ T cells exhibiting Gag$_{241-249}$-specific IFN-γ induction were significantly higher than in naive controls at week 2 (Fig. 3B) and week 12. Gag$_{241-249}$-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 SIVGP1 containing the genes encoding SIVmac239 Gag, Pol, Vif, Vpx, and a part of Vpr and measured the frequencies of CD8$^+$ T cells responding to SIVGP1-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 Gag$_{206-216}$-specific CD8$^+$ T-cell recognition at week 5, implicating Gag$_{206-216}$-specific CD8$^+$ T-cell responses (rather than Gag$_{241-249}$-specific CD8$^+$ T-cell responses) in viral control. In the present study, however, five of six Gag$_{236-250}$-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 Gag$_{241-249}$-specific CD8$^+$ T-cell responses in the acute phase play an important role in this vaccine-based SIV control.

Higher Gag$_{241-249}$-specific effector memory CD8$^+$ T-cell frequencies in vaccinees. We then examined Gag$_{241-249}$-specific CD8$^+$ T-cell frequencies in these macaques by using PE-conjugated Gag$_{241-249}$-A*90120-5 tetramers. In group III animals, Gag$_{241-249}$-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 Gag$_{241-249}$-specific tetramer$^+$ CD95$^+$ CD28$^-$ CD8$^+$ T cells in group III than in naive controls (Fig. 5C). These results suggest efficient responses of Gag$_{241-249}$-specific CD8$^+$ T cells with effector function in the acute phase in group III animals.

Gag$_{241-249}$-specific cytolytic CD8$^+$ T-cell responses in vaccinees. To further investigate the cytolytic quality of Gag$_{241-249}$-specific CD8$^+$ T-cell responses after a challenge, we examined
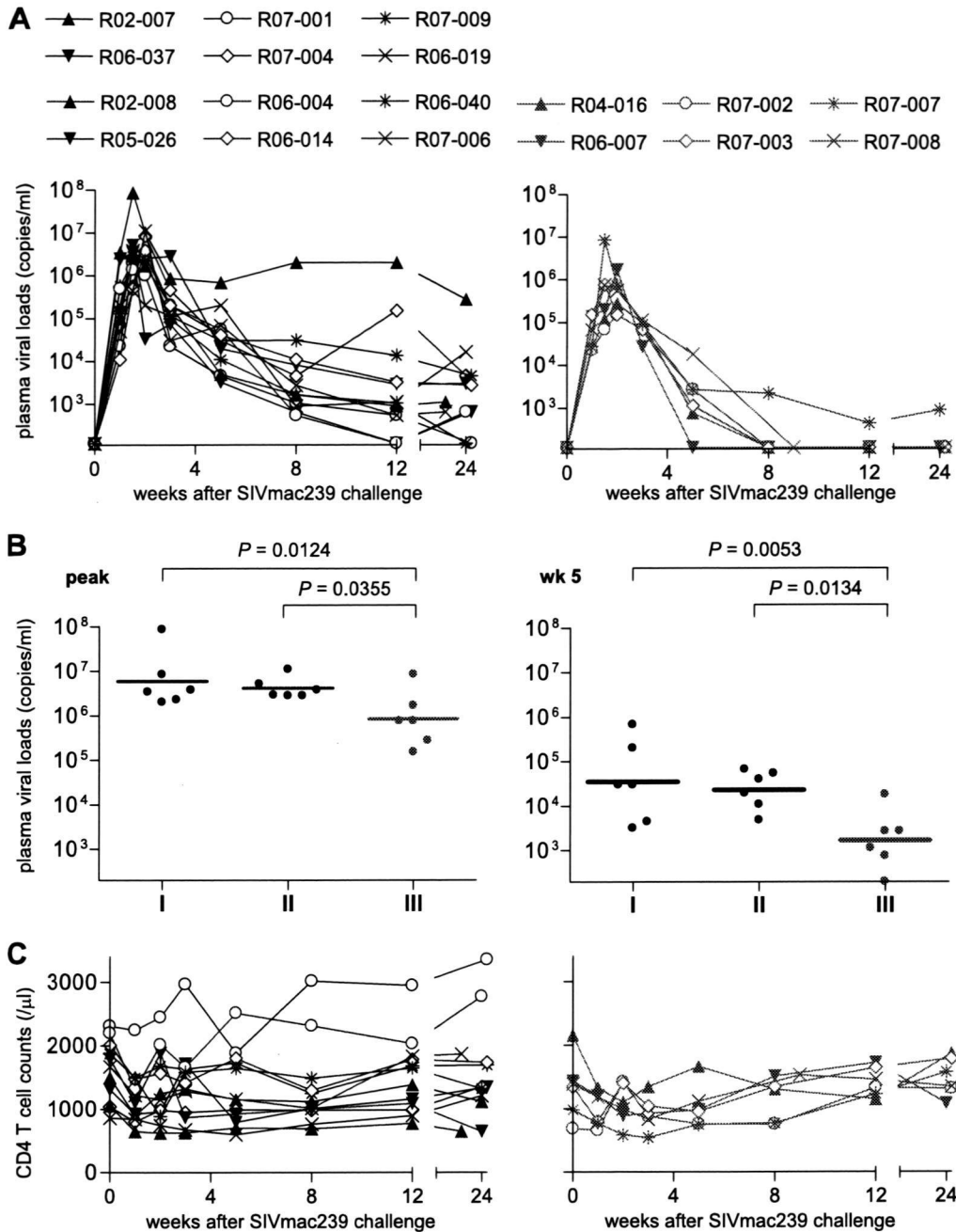
FIG. 2. Plasma viral loads and peripheral CD4$^+$ T-cell counts after a SIV challenge. (A) Changes in plasma viral loads (SIV *gag* RNA copies/ml plasma) in unvaccinated group I animals (black lines in the left panel), control-vaccinated group II animals (blue lines in the left panel), and Gag$_{236-250}$-vaccinated group III animals (red lines in the right panel) after a SIVmac239 challenge. Plasma viral loads were determined as described previously (27). The lower limit of detection is approximately 4×10^2 copies/ml. (B) Comparisons of plasma viral loads in groups I ($n = 6$), II ($n = 6$), and III ($n = 6$) at the peak (left panel) and at week 5 (right panel). The bar indicates the geometric mean of each group. Viral loads at the peak and at week 5 in group III were significantly lower than in group I ($P = 0.0124$ at the peak and $P = 0.0053$ at week 5) and group II ($P = 0.0355$ at the peak and $P = 0.0134$ at week 5). There were no significant differences between groups I and II either at the peak or at week 5 ($P = 0.6047$ at the peak and $P = 0.6536$ at week 5). Set point viral loads in group III were significantly lower than those in group I and group II at week 12 by nonparametric analysis ($P = 0.3939$ between I and II, $P = 0.0152$ between I and III, and $P = 0.0152$ between II and III; $P = 0.1797$ between I and II, $P = 0.0260$ between I and III, and $P = 0.0411$ between II and III around week 24). (C) Changes in peripheral CD4$^+$ T-cell counts (per µl) in groups I (black lines) and II (blue lines) in the left panel and in group III (red lines) in the right panel after a SIVmac239 challenge.

Gag$_{241-249}$-specific induction of CD107a (a degranulation marker), which is related to cytolytic activity (21, 38), in CD8$^+$ T cells at week 2. Frequencies of CD8$^+$ T cells exhibiting Gag$_{241-249}$-specific induction of CD107a, as well as IFN-γ,

within the CD8$^+$ T-cell pool were significantly higher in group III than in naive controls ($P = 0.0249$ by unpaired *t* test) (Fig. 6). One animal, R04-016, in group III did not show Gag$_{241-249}$-specific CD107a$^+$ IFN-γ$^+$ CD8$^+$ T-cell responses, but further

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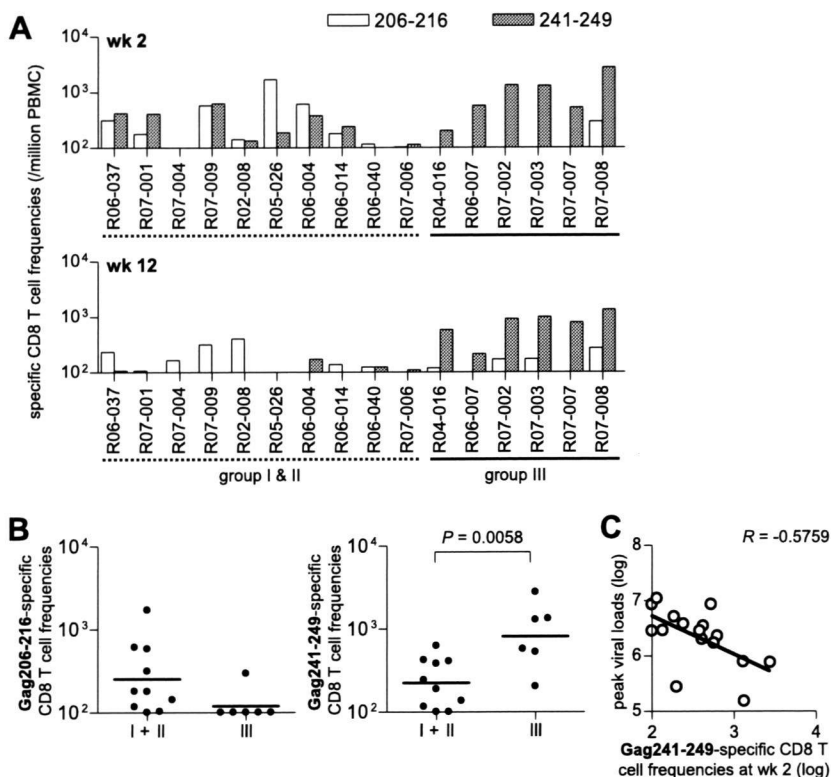
FIG. 3. Gag epitope-specific CD8$^+$ T-cell frequencies after a SIV challenge. (A) Frequencies of CD8$^+$ T cells (per million PBMCs) showing Gag$_{206-216}$-specific (open boxes) or Gag$_{241-249}$-specific (closed boxes) IFN-γ induction in naive controls and group III macaques at week 2 (upper panel) and week 12 (lower panel). (B) Comparison of the Gag$_{206-216}$-specific (left panel) or Gag$_{241-249}$-specific (right panel) CD8$^+$ T-cell frequencies in naive controls ($n = 10$) and group III animals ($n = 6$) at week 2. The bar indicates the geometric mean of each group. Frequencies of Gag$_{241-249}$-specific ($P = 0.0058$) but not Gag$_{206-216}$-specific ($P = 0.0922$) CD8$^+$ T cells in group III were significantly higher than in naive controls. The Gag$_{241-249}$-specific frequencies at week 12 in group III were significantly higher than those in naive controls ($P < 0.0001$). (C) Analysis of the correlation between Gag$_{241-249}$-specific CD8$^+$ T-cell frequencies (log) at week 2 and peak plasma viral loads (log). An inverse correlation is shown ($P = 0.0196$, $R = -0.5759$). Samples from macaques R02-007 and R06-019 in group I were unavailable for this analysis.

analysis revealed that this animal had Gag$_{241-249}$-specific granzyme B$^+$ IFN-γ^+ CD8$^+$ T cells. Indeed, group III animals had significantly higher frequencies of Gag$_{241-249}$-specific IFN-γ^+ CD8$^+$ T cells producing CD107a, granzyme B, or perforin ($P = 0.0076$; data not shown). These results indicate efficient induction of Gag$_{241-249}$-specific CD8$^+$ T cells with higher cytolytic activity in the acute phase in group III animals.
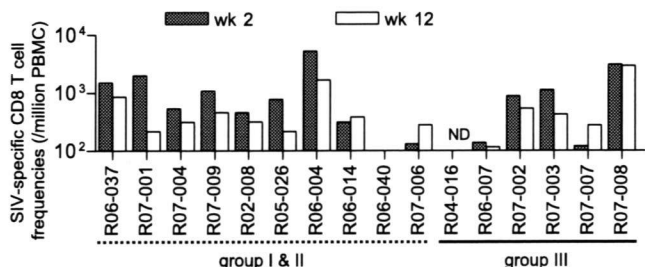

FIG. 4. SIV-specific CD8$^+$ T-cell frequencies after a SIV challenge. SIV-specific CD8$^+$ T-cell frequencies (per million PBMCs) in naive controls and group III macaques at week 2 (closed boxes) and week 12 (open boxes) are shown. ND, not determined.

DISCUSSION

In the present study, induction of CD8$^+$ T cells specific for a single Gag$_{241-249}$ epitope by prophylactic vaccination resulted in a significant reduction of plasma viral loads after a SIV challenge. Even if vaccines are designed to express multiple antigens, of the vaccine-induced CD8$^+$ T cells generated, at most one or only a few epitope-specific cells may recognize the incoming HIV because of viral diversity and host MHC polymorphisms (10). Our finding, however, implies that even a CD8$^+$ T-cell memory response to a single epitope which can recognize the incoming HIV could facilitate HIV control.

Group III macaques showed more effective CD8$^+$ T-cell responses than did naive controls after a SIV challenge. Our previous trial of a vaccine inducing Gag-specific T-cell responses resulted in SIV control in *90-120-Ia*-positive macaques with rapid selection of the GagL216S mutation escaping from Gag$_{206-216}$-specific CD8$^+$ T-cell recognition at week 5 (27). In contrast, the Gag$_{236-250}$ vaccination resulted in SIV control without *gag* mutation selection over 5 weeks in the present study, reflecting the fact that, rather than Gag$_{206-216}$-specific CD8$^+$ T-cell responses, dominantly induced Gag$_{241-249}$-specific CD8$^+$ T-cell responses played a central role in the reduc-