specific susceptibility to infectious disease might be associated with the differences in gene functions linked to the defense against infectious diseases, which would lead the differences in defense mechanisms mounted against invading pathogens. Natural selection pressure has advanced the species-specific evolution in the susceptibility genes for infectious diseases. In fact, dozens of genes under the control of natural selection in the course of primate evolution have been identified to date. Taking all things into consideration, these genes are candidates for determining the susceptibility to human diseases. In the following sections, we describe the examples.

Comparative Genomics Might Uncover Susceptibility Genes for Human Diseases

TRIM5α plays crucial roles in the intracellular defense against HIV-1,[52] and sequence differences in the SPRY domain of TRIM5α contribute to the differences in anti-HIV-1 activity among primate species.[52] Comparative genomics for TRIM5α shows that this gene has rapidly evolved in the course of primate evolution, and that natural selection has shaped the sequence difference in the SPRY domain. Sequence variations in TRIM5 have been reported to be associated with the susceptibility to HIV-1/AIDS in humans. For another example, APOBEC3G, that also plays an important role in the defense against HIV-1, has been under positive Darwinian selection.[53] A comparison of *APOBEC3G* sequences among primates suggests a rapid evolution of APOBEC3G in the course of primate evolution.

Given that *TRIM5α* and *APOBEC3G* are the genes associated with the susceptibility to HIV/AIDS in humans, comparative genomics can be a useful tool for both identifying the candidate genes for controlling the HIV/AIDS susceptibility and evaluating the pathophysiological roles of the genes from an evolutionary point of view. These examples suggest that comparative genomics is a promising approach to identify the susceptibility genes for infectious diseases. Since susceptibilities differ among primates to not only infectious, but also other common diseases, such as Alzheimer's disease and cardiovascular diseases, comparative genomics may be a crucial tool in providing candidate genes to determine the susceptibility for the other diseases as well.

Bioinformatics Tool to Evaluate Functional Impact of Nonsynonymous Variations based on Comparative Genomics

It is estimated that there are 67,000-200,000 common nonsynonymous single nucleotide polymorphisms (SNPs) in the human genome, and that each individual is heterozygous for 24,000-40,000 nonsynonymous SNPs.[54] Because nonsynonymous SNPs would affect the protein function, some of them might be associated with human health and disease. Recently, various computational approaches to assessing the functional significance of

nonsynonymous SNPs have been developed.[55-61] They predict whether an amino acid substitution induced by nonsynonymous SNPs affects protein function based on the comparative genomics, physical properties of amino acids, and/or three-dimensional (3D) structures of proteins. The main features of the representative methods are summarized in Table 21.5. These programs are also useful to estimate the significance of functional impact induced by nonsynonymous mutations in the genes for single-gene disorders.

Some of the programs are sequence-based amino acid substitution prediction method based on the comparative genomics, which are founded on the concept that the amino acid substitutions affecting protein function tend to occur at conserved evolutionary sites. Such conserved sites, as described in the previous sections, have come under the control of negative (purifying) selection, and are considered to be important for protein function. A multiple sequence alignment among the homologous sequences indicates the conserved sites throughout the course of evolution. The sequence-based amino acid substitution prediction method scores the levels of amino acid substitution based on the amino acids appearing in the multiple alignments, and the severity of the amino acid change based on the physical properties. It has been reported that 25-35 percent of nonsynonymous SNPs are predicted to affect the protein function by the

Table 21.5: The main features of representative prediction methods used to evaluate the impact of nonsynonymous SNPs on protein function

Method	*Web site*	
SIFT[55]	http://sift.jcvi.org/	Sequence-based prediction method using position-specific scoring matrices with Dirichlet priors
PolyPhen[56]	http://coot.embl.de/PolyPhen/	Structure/sequence-based prediction method
SNPs3D[57]	http://www.snps3d.org/	Structure/sequence-based prediction method
PANTHER PSEC[58]	http://www.pantherdb.org/tools/csnpScoreForm.jsp	Sequence-based prediction method using PANTHER Hidden Markov Model families
PMUT[59]	http://mmb2.pcb.ub.es:8080/PMut/	Structure/sequence-based prediction method
TopoSNP[60]	http://gila.bioengr.uic.edu/snp/toposnp/	Structure/sequence-based prediction method A database of topographic mapping of SNPs
MAPP[61]	http://mendel.stanford.edu/SidowLab/downloads/MAPP/index.html	Sequence-based prediction method

most widely used prediction methods. Currently, automated prediction methods are being applied on a genome-wide scale, which might accelerate the findings of human disease susceptibility genes in the near future. However, it has been pointed out that there are limitations to the current prediction methods. For example, Thomas et al[62] have reported that the current method may not be useful for identifying certain nonsynonymous SNPs involved in human common diseases. In any events, however, it is reasonable to expect further progress in the field of bioinformatics using these and related methods.

CONCLUSION

In this chapter, we introduced concepts and methods for evaluating the biological significance of homologues sequences, especially focusing on the methods that are based on the theory of natural selection. Although the comparative genomics are not definitive in determining the biological significance of conserved sequences, this approach is nonetheless highly useful as the first step for identifying and characterizing functional regions in the genome. We have introduced here only a small part of comparative genomics. We recommend referring to a number of reviews to cover the wide variety of the features of comparative genomics.[1, 11-13]

Recent rapid progress in the field of bioinformatics and sequencing technology has brought about a breakthrough in the comparative genomic analysis. It is therefore expected that further progress in the comparative genomics will provide a stream of novel insight into health and disease in humans.

ACKNOWLEDGMENTS

This work was supported in part research grants from the Ministry of Health, Labor and Welfare, Japan, the Japan Health Science Foundation, the program of Founding Research Centers for Emerging and Reemerging Infection Disease, the Japan Health Science Foundation, the program of Research on Publicly Essential Drugs and Medical Devices, Grant-in-Aids for Scientific research from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT), Japan, grants for Indo-Japan collaboration research from DST and JSPS, and a grant from Heiwa Nakajima Foundation.

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JOURNAL OF VIROLOGY, June 2009, p. 5514–5524 Vol. 83, No. 11
0022-538X/09/$08.00+0 doi:10.1128/JVI.00145-09
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Polyfunctional CD4$^+$ 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 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.

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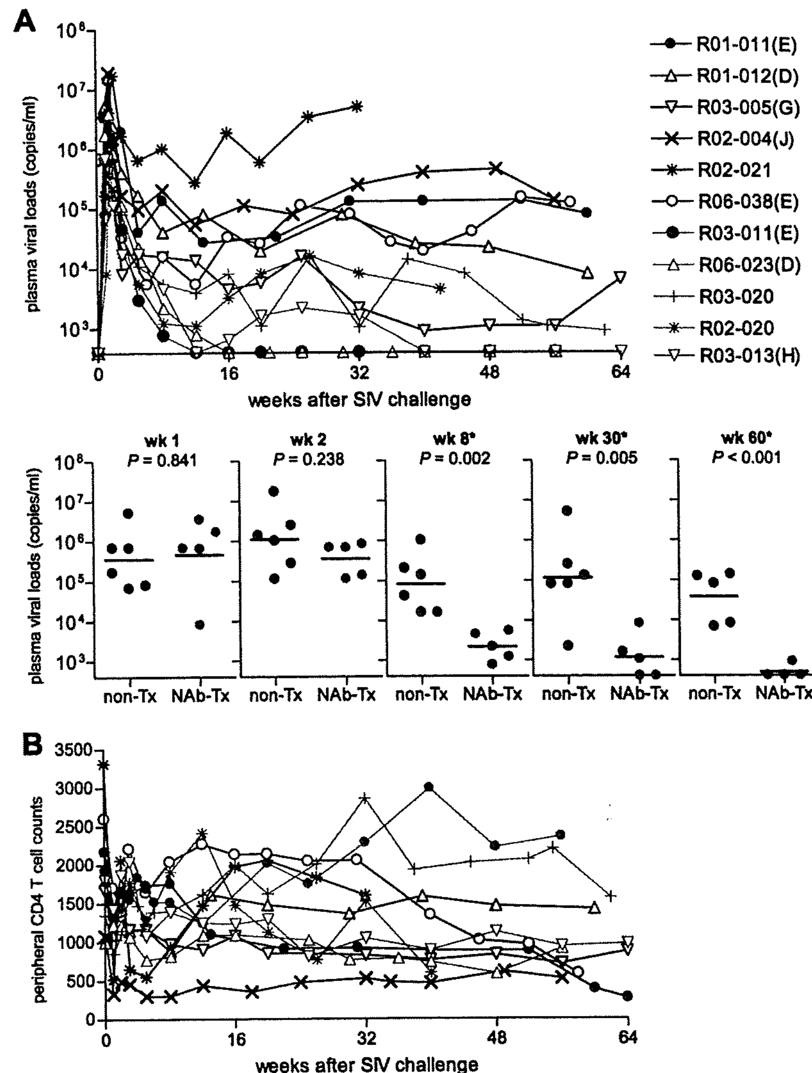

FIG. 1. Follow up of NAb-immunized macaques. (A) Plasma viral loads (SIV *gag* RNA copies/ml plasma) in six unimmunized macaques (black lines) and five NAb-immunized animals (red lines) after SIVmac239 challenge. The plasma viral loads were measured as described previously (29). The lower limit of detection 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-

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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

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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.

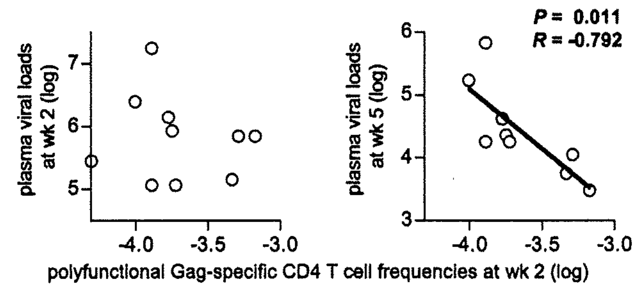

FIG. 3. Analysis of correlation between polyfunctional Gag-specific CD4$^+$ T-cell frequencies (log) at week (wk) 2 and plasma viral loads (log) at week 2 (left) or week 5 (right). Inverse correlation is shown on the right ($P = 0.011$; $R = -0.792$), but not on the left ($P = 0.694$; $R = -0.143$).

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 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

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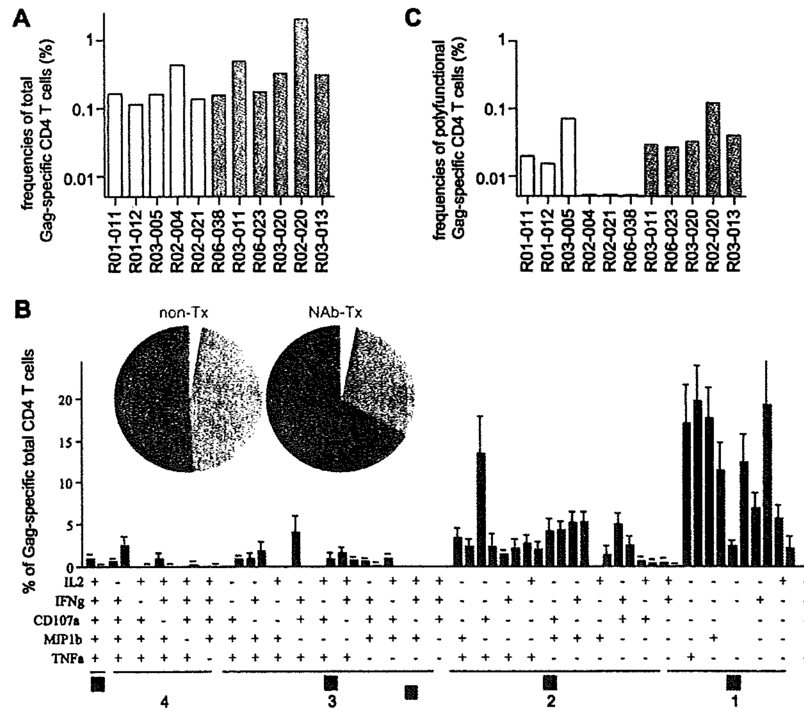

FIG. 4. Gag-specific CD4⁺ T-cell responses in the chronic phase. PBMCs around week 30 postchallenge were stimulated with p55, and specific induction of IFN-γ, TNF-α, IL-2, MIP-1β, and CD107a in CD4⁺ T cells was examined. (A) Frequencies of total Gag-specific CD4⁺ T cells. (B) Percentages of cells exhibiting 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,

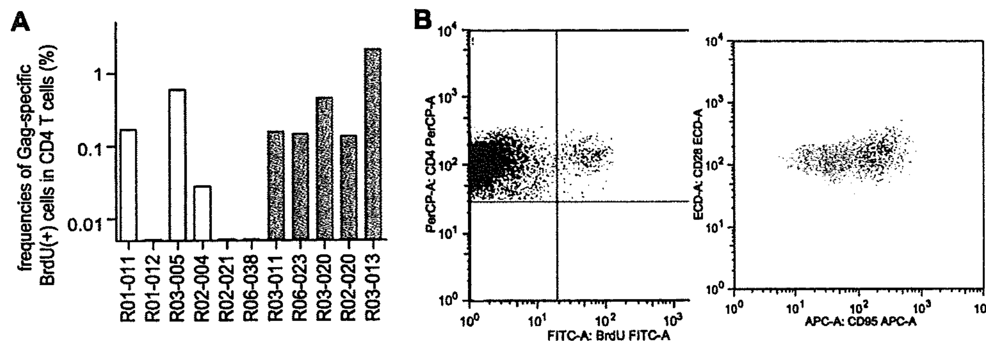

FIG. 5. Gag-specific CD4⁺ T-cell proliferative responses in the chronic phase. PBMCs around week 30 postchallenge were stimulated with p55, and specific uptake of BrdU in CD4⁺ T cells was examined. (A) Frequencies of Gag-specific BrdU⁺ CD4⁺ T cells in total CD4⁺ T cells. The frequencies in NAb-immunized 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.

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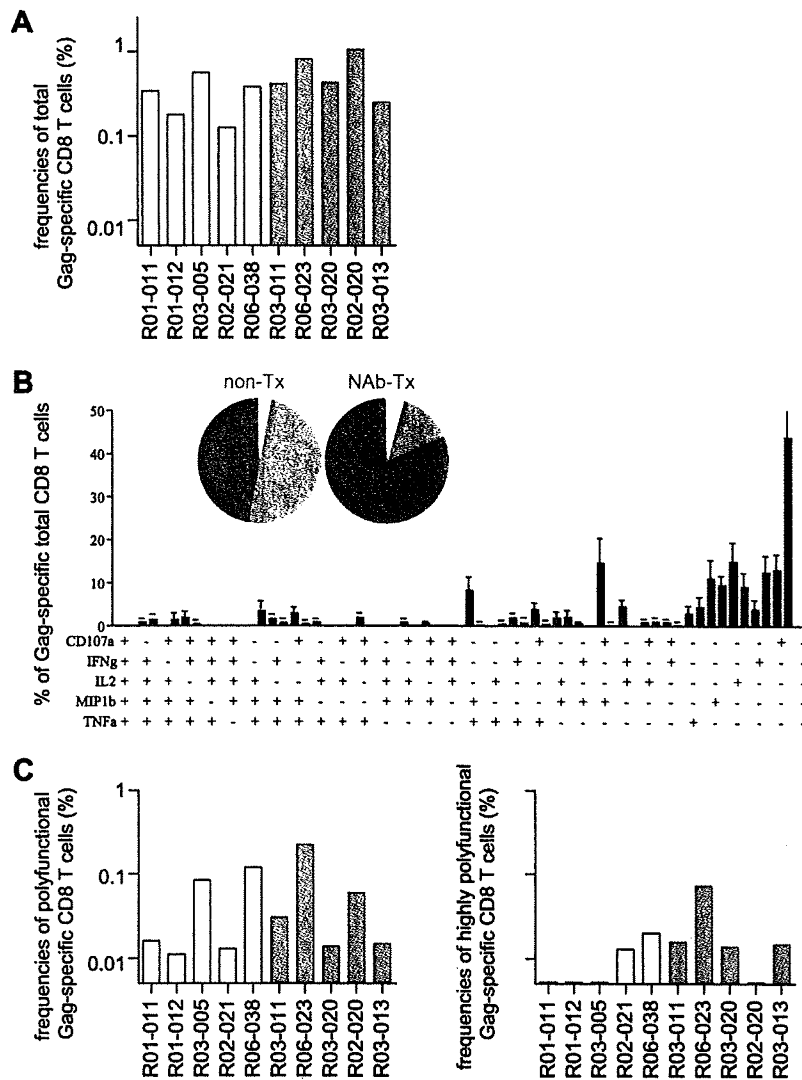

FIG. 6. Gag-specific CD8⁺ T-cell responses in the acute phase. PBMCs at week 2 were stimulated with p55, and specific induction of IFN-γ, TNF-α, IL-2, MIP-1β, and CD107a in CD8⁺ T cells was examined. (A) Frequencies of total Gag-specific CD8⁺ T cells. (B) Percentages of cells exhibiting Gag-specific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

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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 animals, 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.

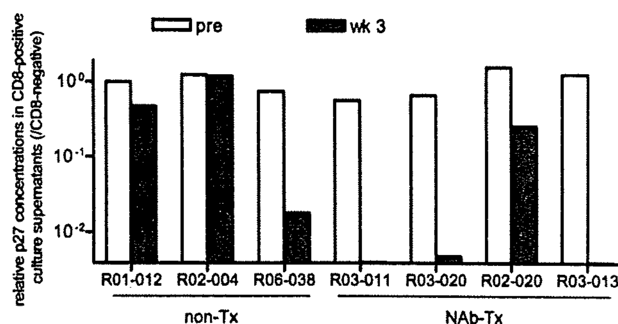

FIG. 7. Anti-SIV efficacy in vitro of CD8$^+$ cells. PBMC-derived CD8$^-$ (target) cells infected with SIVmac239 were cultured alone (no CD8) or cocultured with autologous PBMC-derived CD8$^+$ (effector) cells obtained prechallenge (pre) or at week 3 postchallenge (wk 3) at an E/T ratio of 1:4. The results 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.

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-

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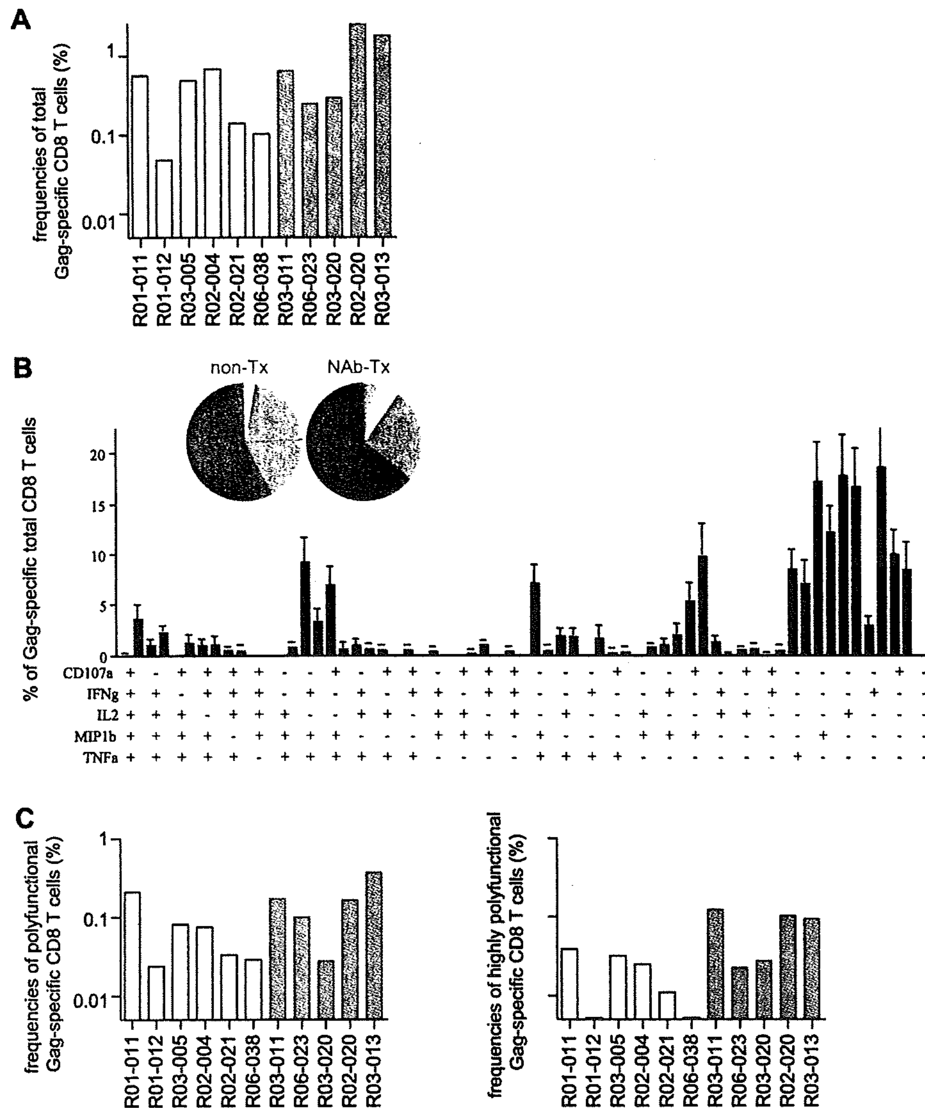

FIG. 8. Gag-specific CD8$^+$ T-cell responses in the chronic phase. PBMCs around week 30 postchallenge were stimulated with p55, and specific induction of IFN-γ, TNF-α, IL-2, MIP-1β, and CD107a in CD8$^+$ T cells was examined. (A) Frequencies of total Gag-specific CD8$^+$ T cells. (B) Percentages of 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$^+$

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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-specific $CD8^+$ 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.

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JOURNAL OF VIROLOGY, Sept. 2009, p. 9339–9346 Vol. 83, No. 18
0022-538X/09/$08.00+0 doi:10.1128/JVI.01120-09
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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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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 $Gag_{241-249}$ 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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▽ Published ahead of print on 8 July 2009.

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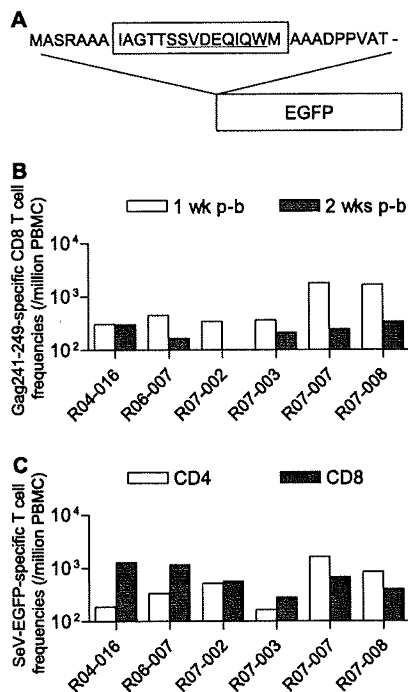

FIG. 1. $Gag_{241-249}$-specific $CD8^+$ T-cell induction by prophylactic vaccination. (A) Schema of the cDNA construct encoding the $Gag_{236-250}$-EGFP fusion protein. A DNA fragment that encodes a 31-mer peptide including the $Gag_{236-250}$ sequence was introduced into the 5′ end of the EGFP cDNA. (B) $Gag_{241-249}$-specific $CD8^+$ T-cell frequencies 1 (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. p$Gag_{236-250}$-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 p$Gag_{236-250}$-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 $\alpha = 0.05$ (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

$Gag_{241-249}$-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 (p$Gag_{236-250}$-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 p$Gag_{236-250}$-EGFP DNA injection, followed by a single intra-

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