

表1 SubtypeB (N=132) におけるProteaseの薬剤耐性変異に対するpr55Gagの変異

Protease mutation	gag mutation							
	MA (p17)	CA (p24)			p7	p1	p6	
D30N (8)		R264K*	N315T**		M423L**		R452K**	P453L** S473P** S498L** P453V**
M46I (19)	A83V**	S165N*	P255A**	E260D*	T280V*	A431V**		
M46L (9)	A120G**	E203D**	T239S**	I247V*	E260D**	A431V**		
V82A (15)	A120G**	Y132F**	E203D*	T239S*	V323I*	A431V**	I437V**	T469K*
L90M (29)	V7I*	A83V**	Y132F**				I437V*	L449V* S491T*
L10I (30)	Y132F*		P255A*	T303V*		A431V**		L449V*
L10F (6)								L449P*
L10F (7)	Q65H*	S165N**	E203D**	I247V**		A431V**		G466R*
K20R (7)	V7I*							
L24I (6)	A120G**	E203D**	I247V**	V362I*		A431V**		L483M*
L33F (8)	A120G**	E203D**	I247V*			A431V**		
M36I (43)	R76K*							
F53L (6)	Y132F*	S176A**						
I54V (16)	A120G**	Y132F**	E203D*	T239S**		A431V**		
A71T (9)	V7I**	L268M*						S498L*
A71V (32)	A83V**	Y132F*	S176A*				I437V**	
V77I (56)		P255A**						K481E*
N88D (7)	Q69K*	R264K**	L268M*	N315T**	M423L**		R452K**	P453L** S498L**

\* :p<0.01

\*\* :p<0.001

下線:Mutation in cleavage site

### Ⅲ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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#### IV. 研究成果の刊行物・別刷

# Induction of Positive Cellular and Humoral Immune Responses by a Prime-Boost Vaccine Encoded with Simian Immunodeficiency Virus *gag/pol*<sup>1</sup>

Kenji Someya,\* Yasushi Ami,<sup>†</sup> Tadashi Nakasone,\* Yasuyuki Izumi,\* Kazuhiro Matsuo,\* Shigeo Horibata,\* Ke-Qin Xin,<sup>‡</sup> Hiroshi Yamamoto,<sup>§</sup> Kenji Okuda,<sup>‡</sup> Naoki Yamamoto,\* and Mitsuo Honda<sup>2\*</sup>

It is believed likely that immune responses are responsible for controlling viral load and infection. In this study, when macaques were primed with plasmid DNA encoding SIV *gag* and *pol* genes (SIV*gag/pol* DNA) and then boosted with replication-deficient vaccinia virus DIs recombinant expressing the same genes (rDIsSIV*gag/pol*), this prime-boost regimen generated higher levels of Gag-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses than did either SIV*gag/pol* DNA or rDIsSIV*gag/pol* alone. When the macaques were i.v. challenged with pathogenic simian/HIV, the prime-boost group maintained high CD4<sup>+</sup> T cell counts and reduced plasma viral loads up to 30 wk after viral challenge, whereas the rDIsSIV*gag/pol* group showed only a partial attenuation of the viral infection, and the group immunized with SIV*gag/pol* DNA alone showed none at all. The protection levels were better correlated with the levels of virus-specific T cell responses than the levels of neutralization Ab responses. These results demonstrate that a vaccine regimen that primes with DNA and then boosts with a replication-defective vaccinia virus DIs generates anti-SIV immunity, suggesting that it will be a promising vaccine regimen for HIV-1 vaccine development. *The Journal of Immunology*, 2006, 176: 1784–1795.

The primary goals of any prophylactic HIV vaccine are to induce HIV-specific immune responses capable of preventing the malfunctioning of immune systems and to limit viral transmission due to replication. Clinical studies have demonstrated that CTL immune responses are associated with the reduction of plasma viral load (1, 2) and can control disease progression (3, 4). Replication of pathogenic SIV in vivo has also been shown to be controlled in the macaque model by CD8<sup>+</sup> T cell responses (5). Because amino acid sequences of Gag and Pol of HIV-1 proteins are relatively conserved, cross-clade and broad CTL responses targeting those proteins have been observed in both HIV-infected and HIV-exposed individuals, even if the latter group had not become infected (6–8). Thus, one recent focus of HIV vaccine research has been to elicit more protective antiviral immune responses by enhancing the expression levels of HIV-1 Ags of Gag and Pol using a safe vaccine vector.

Recently, several prime-boost regimens consisting of a DNA prime and a recombinant poxvirus boost targeting the immunodeficiency virus have been reported to generate higher levels of HIV-

specific T cell immune responses than regimens relying on DNA or recombinant poxvirus vaccine alone (9, 10). In efficacy trials of such heterologous prime-boost vaccines, an SIV Ag encoding DNA prime and a boost of recombinant modified vaccinia virus Ankara (MVA)<sup>3</sup> elicited effective anti-SIV immunity and controlled infection of the nonpathogenic simian-HIV (SHIV) strain as well as of the pathogenic strain SHIV-89.6P in macaques (11–13) by effectively inducing CD8<sup>+</sup> CTL immunities. Various poxvirus vectors, i.e., an avipox virus, a canarypox virus, a fowlpox virus, a substrain of vaccinia Copenhagen (NYVAC), and MVA, have been evaluated for their usefulness, either alone or in combination with other vaccine modalities (14–18). To be useful, these vaccine vectors must, of course, be safe. The currently widely used MVA, which was developed toward the end of the campaign to eradicate small pox, has been effectively and safely used in >100,000 people as a small pox vaccine (19). MVA-based recombinant vector has also been reported to be safe in animals (20, 21). Lately, we have developed a replication-defective vaccinia virus DIs strain as a vaccine vector (22, 23). The DIs strain, generated by a 1-day-old egg passage of the DIE strain (24), has been proven safe (25, 26). We also suggested that a new prime-boost vaccine regimen consisting of SIV*gag/pol* DNA and rDIsSIV*gag/pol* might be useful for the development of an HIV-1 candidate vaccine that could induce strong cellular protective responses in mice (23). Lately, similar DNA/MVA vaccine combinations support the idea that the vaccine induced strong Ag-specific T and B cell responses (27). The prime-boost-vaccinated mice generated higher levels of both Gag-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell immune responses than those vaccinated with either DNA or rDIs alone. When such mice were challenged with SIV *gag/pol* expressing

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<sup>3</sup>Abbreviations used in this paper: MVA, modified vaccinia virus Ankara; rDIsSIV*gag/pol*, recombinant DIs expressing SIV *gag* and *pol*; SFC, spot-forming cell; SHIV, simian-human immunodeficiency virus; SIV*gag/pol* DNA, plasmid DNA encoding SIV *gag* and *pol* genes; TCID<sub>50</sub>, 50% tissue culture infectious doses.

wild-type recombinant vaccinia virus, viral replication in the ovaries was controlled even in the absence of anti-DIs immunity. These results suggest that the new vaccine regimen, consisting of a DNA prime and a vaccinia virus DIs boost, safely and effectively elicits anti-immunodeficiency viral immunity.

In this study, we evaluated the vaccine efficacy of the prime-boost DNA/DIs vaccine encoding the *gag/pol* gene against a challenge with a highly pathogenic SHIV using 19 macaques. We hypothesize that the efficacy is mediated not only by the effect of virus-specific cellular immunity, but also by the effect of neutralization Ab responses against the challenged virus.

## Materials and Methods

### Animals

Nineteen female adult cynomolgus macaques (*Macaca fascicularis*) were purchased from Japan SLC. The macaques were fed and cared for in accordance with the standard operating procedure approved by the Ministry of Education, Culture, Sports, Science, and Technology of Japan. The study was performed in the P3 facility under guidelines established by the laboratory biosafety manual of the World Health Organization (28).

### Preparation of vaccine Ags and challenge virus

Plasmid DNA encoding SIV *gag* and *pol* genes (SIV*gag/pol* DNA) and recombinant DIs expressing the same genes (rDIsSIV*gag/pol*) were prepared as previously described (22, 23, 29). pcDNA3.1<sup>+</sup> and rDIsLacZ were used as controls of plasmid and recombinant viral Ags, respectively. After being immunized according to the protocol, animals were challenged with SHIV-C2/1 (30–32), which was a SHIV-89.6 variant isolated at the peak of initial plasma viremia from an infected cynomolgus macaque (31). The original SHIV strain was provided by Dr. Y. Lu (Harvard AIDS Institute, Cambridge, MA) (33, 34).

### Enumeration of T PBL

Fifty microliters of whole heparinized blood samples were stained with anti-human CD3 (clone HIT3a; BD Pharmingen), anti-human CD4 (clone SK3; BD Biosciences), and anti-human CD8 (clone SK1; BD Biosciences) for 15 min at 4°C. Blood samples were treated with FACS lysing solution for 15 min at 4°C, and then 50  $\mu$ l of Flow Count (Beckman Coulter) was added. A FACSCalibur flow cytometer (BD Biosciences) was used to acquire 5000 CD3-positive, lymphocyte-gated events.

### Intracellular IFN- $\gamma$ cytokine staining

Approximately  $10^6$  of fresh PBMC were incubated with 0.2  $\mu$ M pooled SIV Gag peptides spanning the full length of the Gag protein (AIDS Research and Reference Program, National Institutes of Health) together with 1  $\mu$ g of anti-human CD28 (clone KOL.T-2; Nichirei) and 1  $\mu$ g of anti-human CD49d (clone 9F10; BD Pharmingen) in an appropriate volume of RPMI 1640 supplemented with 10% FBS and antibiotics for 16 h at 37°C. Then brefeldin A (Sigma-Aldrich) was added at 10  $\mu$ g/ml, and the cells were incubated for an additional 4 h. After incubation, the cells were washed, stained with anti-human CD3 (clone HIT3a; BD Pharmingen) and anti-human CD8 (clone SK1; BD Biosciences) or anti-human CD4 (clone SK3; BD Biosciences) for 15 min. The cells were washed and then treated sequentially with FACS-lysing solution (BD Biosciences) and permeabilizing solution (BD Biosciences) for 10 min. The cells were stained with anti-human IFN- $\gamma$ -FITC (clone 45.15; Immuno Tech) for 30 min and fixed with 2% paraformaldehyde solution. A FACSCalibur flow cytometer (BD Biosciences) was used to acquire 20,000 lymphocyte-gated events, which were then analyzed with CellQuest software (BD Biosciences).

### Virus-specific IFN- $\gamma$ ELISPOT assay

An ELISPOT assay was performed following the method developed by Mothe and Watkins of the Wisconsin University Primate Center (35). Ninety-six-well, flat-bottom plates were coated with anti-IFN- $\gamma$  mAb (clone MD-1; U-CyTech-BV) and blocked with 2% BSA in PBS. Fresh PBMC were added to the plate at  $2 \times 10^5$  cells/well in triplicate and then incubated with 0.2  $\mu$ M pooled SIV Gag peptides (AIDS Research and Reference Program) for 16 h at 37°C. Gold-labeled anti-biotin IgG solution (U-CyTech-BV) was added to the washed plates, which were then incubated for 1 h at 37°C. Individual spot-forming cells (SFC) were counted using the KS ELISPOT compact system (Zeiss) after a 15-min reaction with an activator mix (U-CyTech-BV). An SFC was defined as a large black spot with a fuzzy border (34).

### Abs to SIV Gag p27 and SHIV 89.6P Env

SIV Gag- and SHIV Env-specific IgG Ab end-point titers of the macaques' sera were measured by ELISA as previously described (23, 27, 30). All samples were run in triplicate at several dilutions. In brief, 96-well ELISA plates were coated with 0.3  $\mu$ g of SIV p27 Gag (Advanced Biotechnologies) or 0.2  $\mu$ M pooled SHIV 89.6P Env peptides (AIDS Research and Reference Program) per well. Heat-inactivated sera were serially diluted, then added to the ELISA plates. Gag- and Env-specific Abs bound to the Ags were captured with alkaline phosphatase-labeled goat anti-mouse IgG (EY Laboratories) and *p*-nitrophenyl-phosphate disodium substrate (Invitrogen Life Technologies).

The SHIV Env-specific neutralization Ab responses induced by challenge with SHIV were analyzed as previously described (28). In brief, 10  $\mu$ g/ml purified macaque IgG was incubated with 100 50% tissue culture infectious doses (TCID<sub>50</sub>) of SHIV-C2/1, then cultured in M8166 cells. The result was compared with parallel cultures to which preimmune IgG had been added. Neutralization was expressed as the percent inhibition of SIV Gag production in the culture supernatants. Anything >20% of inhibition was considered to be an efficient neutralization response.

### Quantitation of plasma viral load

Quantitation of SHIV genomic RNA copies in plasma samples was performed by real-time PCR with a TaqMan assay kit (PerkinElmer Applied Biosystems) and a PRISM 7700 sequence detection system (PerkinElmer Applied Biosystems) as previously described (30). Genomic RNA extracted from plasma samples and SIVmac239 (RNA standard;  $5.4 \times 10^4$  RNA copies) were subjected to RT-PCR using an SIVmac239–1224 forward, SIVmac239–1326 reverse primer pair and an FAM-SIV-1272T probe. RNA copy numbers from plasma samples were calculated from the standard curve. Data were expressed as RNA copies per milliliter of plasma.

### Flow cytometric detection of various subpopulations in CD4<sup>+</sup> T cells

Approximately  $10^6$  fresh PBMC were stained with anti-human CD4 (clone SK3; BD Biosciences), anti-human CD29 (clone 4B4; Beckman Coulter), and anti-human CD45RA (clone 5H9; BD Pharmingen) or with anti-human CD4 (clone  $\nu$ -TH/1; Nichirei) and anti-human CD28 (clone KOL.T-2, Nichirei). A FACSCalibur (BD Biosciences) was used to acquire 10,000 lymphocyte-gated events, which were then analyzed with CellQuest software.

### Statistical analysis

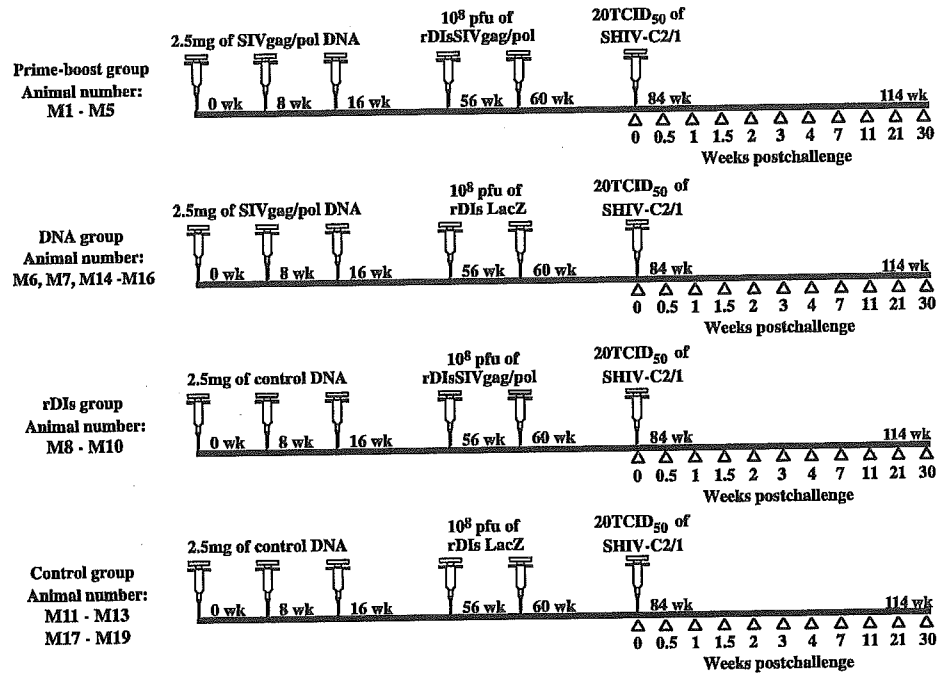
Data are expressed as the mean  $\pm$  SD. The data analysis was conducted using the StatView program (SAS Institute), and all reported *p* values are two-sided. Comparisons between groups were performed using the Kruskal-Wallis *H* test, followed by the Student-Newman-Keuls correction. Correlations between protection and immune levels were analyzed using Spearman's rank correlation test. A value of *p* < 0.05 was considered significant.

## Results

### Immunization protocol

Plasmid DNA and the recombinant vaccinia DIs viruses with the inserted *gag/pol* region of SIVmac239 were constructed as previously described (23). Southern blotting confirmed that all plasmids and viruses had the expected genomic structures, whereas Gag-specific Western blots verified the *in vitro* expression of SIV Gag protein in rDIsSIV*gag/pol*-infected chick embryo fibroblasts (data not shown). In this study we opted to use the three-injection regimen for DNA immunization. Because we found that both the three- and five-injection DNA immunization strategies resulted in similar levels of T cell immunities (23). A total of 19 cynomolgus macaques were divided into four groups (Fig. 1). Group 1 macaques (prime-boost group of five macaques numbered M1 to M5) received three i.m. injections (2.5 mg) of each type of SIV*gag/pol* DNA at 8-wk intervals, followed by two injections of  $10^8$  PFU of rDIsSIV*gag/pol*. Group 2 macaques (DNA group of five macaques numbered M6, M7, and M14 through M16) received three i.m. injections of the same dose of each type of SIV*gag/pol* DNA at

**FIGURE 1.** Scheme for immunization and viral challenge. Nineteen macaques were divided into four experimental groups and immunized with 2.5 mg of plasmid DNA at weeks 0, 8, and 16, then immunized with  $10^8$  PFU of rDIs at weeks 56 and 60. Twenty-four weeks after the final immunization, macaques were challenged with 20 TCID<sub>50</sub> of SHIV.



8-wk intervals, followed by two injections of  $10^8$  PFU of rDIs-LacZ. Group 3 macaques (rDIs group of three macaques numbered M8 through M10) received three i.m. injections of control DNA pcDNA3.1<sup>-</sup> at 8-wk intervals, followed by two injections of  $10^8$  PFU of rDIsSIVgag/pol. Group 4 (control group of six macaques numbered M11 through M13 and M17 through M19) received three i.m. injections of control DNA, followed by two injections of  $10^8$  PFU of rDIsLacZ. Twenty-four weeks after the second booster inoculation, the macaques were i.v. challenged with 20 TCID<sub>50</sub> of pathogenic SHIV-C2/1, which were obtained by serum passages of SHIV-89.6. The effects of prime-boost vaccination with DNA and vaccinia DIs on protective immune induction were monitored for 30 wk, then animals were autopsied.

#### *Induction of cellular and humoral immune responses specific for SIV Gag*

We first analyzed the induction of cellular immunity by detecting the SIV Gag-specific IFN- $\gamma$  ELISPOT activities of macaque PBMC after the first and third DNA primings and the first boosting of recombinant DIs in each animal (Fig. 2). A regimen of three consecutive immunizations with SIVgag/pol DNA induced 3- to 4-fold higher IFN- $\gamma$  SFC than did a single immunization in the prime-boost and DNA groups ( $p < 0.05$ ; Fig. 2, A and B). The numbers of IFN- $\gamma$ -producing SFC increased ~8- to 9-fold after booster immunization with rDIsSIVgag/pol in the prime-boost group ( $p < 0.01$ ; Fig. 2C). In contrast, no such increase was seen after booster immunization with rDIsLacZ in the DNA group (Fig. 2C). Macaques immunized with control DNA followed by rDIs-SIVgag/pol (rDIs group) generated higher IFN- $\gamma$  SFC than the DNA group ( $p < 0.01$ ; Fig. 2, B and C). At no point in the course of immunization was Gag-specific IFN- $\gamma$  SFC detected in the control group. Collectively, our findings show that the DNA/rDIs prime-boost immunization efficiently induced immunodeficiency virus-specific ELISPOT activity in macaques.

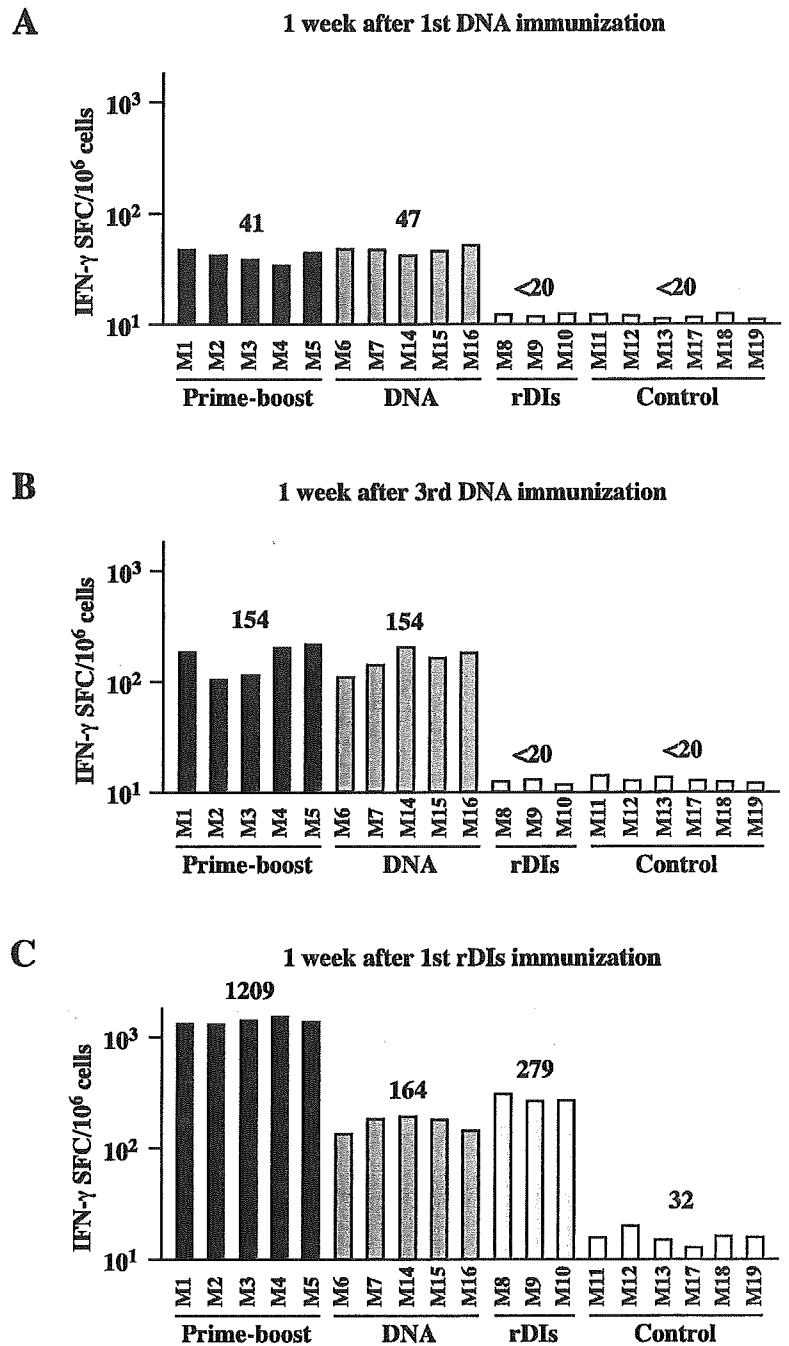
To substantiate the induction of cellular immunity specific for SIV Gag, intracellular IFN- $\gamma$  staining was performed using PBMC after the first booster immunization with rDIs (Fig. 3). Of the four groups tested, the prime-boost group showed the highest frequency

of IFN- $\gamma$ -producing CD4<sup>+</sup> and CD8<sup>+</sup> T cells. The frequencies of Gag-specific IFN- $\gamma$ -producing CD8<sup>+</sup> and CD4<sup>+</sup> T cell responses to Gag peptides in the prime-boost group ranged from 0.51 to 1.22% with an average of 0.82%, and from 0.37 to 0.63% with an average of 0.46%, respectively. The expression of IFN- $\gamma$ -producing CD8<sup>+</sup> and CD4<sup>+</sup> T cells immunized with either SIVgag/pol DNA (average of CD8<sup>+</sup> T cells, 0.095%; average of CD4<sup>+</sup> T cells, 0.015%) or rDIsSIVgag/pol (average of CD8<sup>+</sup> T cells, 0.27%; average of CD4<sup>+</sup> T cells, 0.05%) was apparently weak (Fig. 3). Therefore, as observed for the induction of the SIV Gag-specific ELISPOT activities, the prime-boost group proved to be the most efficient of the four animal groups tested at inducing Ag-specific intracellular IFN- $\gamma$  cytokine staining.

To test for the induction of humoral immunity, we assessed the SIV Gag-specific IgG titers in the animals of each group (Fig. 4). Despite the elevation of Ab titers after the first immunization with SIVgag/pol DNA, no enhanced responses were observed after two serial immunizations with the DNA (Fig. 4A). However, although the titers did not exceed 2000, enhanced Ab responses were observed after booster immunization with rDIsSIVgag/pol. In summary, these results show that the prime-boost vaccine with DNA/rDIs predominantly elicits SIV Gag-specific cellular immune responses in immunized animals.

#### *Enhancement of SIV-specific T cell and humoral immune responses after viral challenge*

Twenty-four weeks after the second immunization with rDIs, macaques were challenged with highly pathogenic SHIV. As shown in Fig. 5A, Gag-specific IFN- $\gamma$  SFC levels decreased on the day of challenge in all vaccinated groups, but the increase observed in the numbers of the SFC after SHIV challenge varied among the groups. The most pronounced increase was seen in the prime-boost group, with the average number of Gag-specific IFN- $\gamma$ -producing cells increasing from 288/million PBMC on the day of challenge to 1124 ( $p < 0.01$ ) 3 days after challenge. The DNA group increased from an average of 104 to 282 ( $p < 0.01$ ), and the rDIs group from 114 to 347 ( $p < 0.05$ ). No significant increases were noted in the control group.



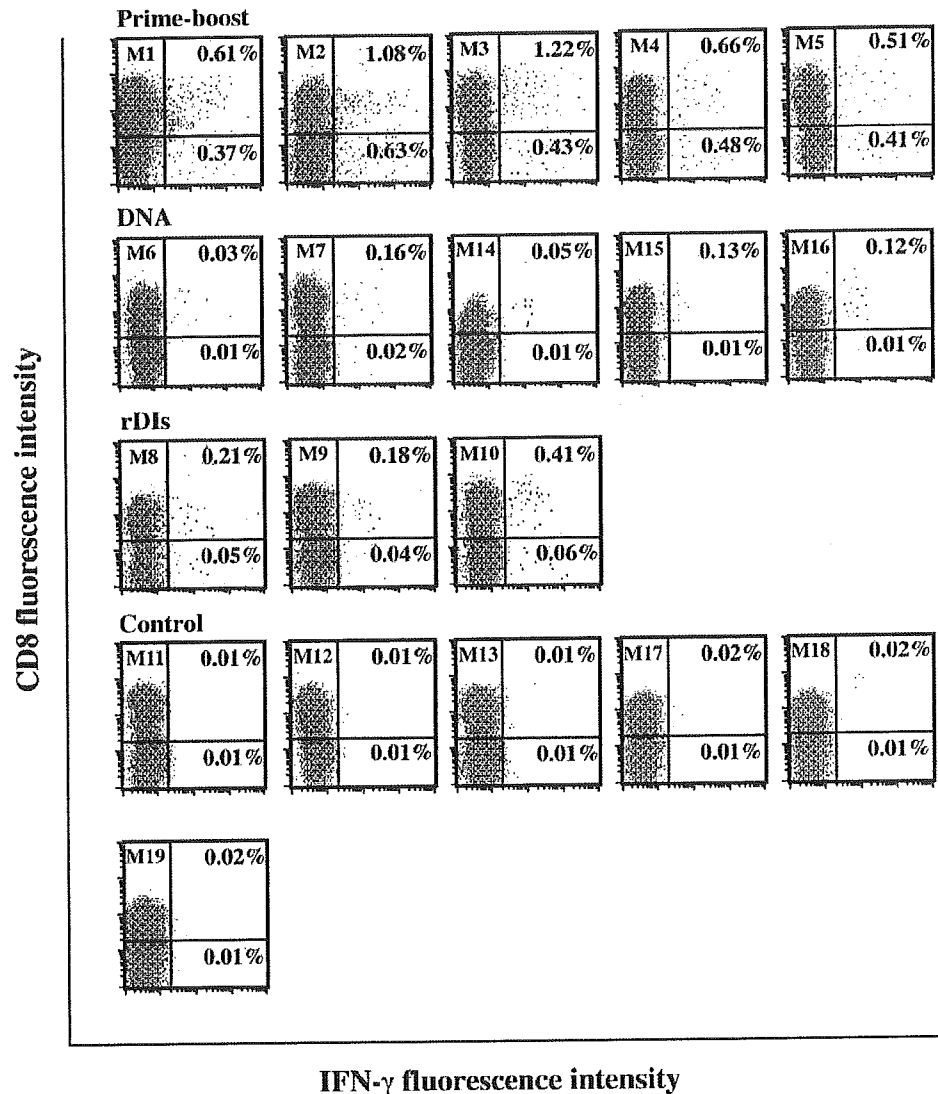
**FIGURE 2.** Frequency of SIV Gag-specific IFN- $\gamma$ -producing cells in immunized macaques. Values are provided at 1 wk after the first DNA immunization (A), 1 wk after the third DNA immunization (B), and 1 wk after rDIs immunization (C). The numbers above the data bars represent the geometric means of the SFC levels in each group. Experimental groups and animal numbers are indicated at the bottom of the graph.

Intracellular IFN- $\gamma$  staining of CD8<sup>+</sup> and CD4<sup>+</sup> T cells was also performed to assess any enhancement in immunodeficiency virus-specific immune responses (Fig. 5B). On the day of challenge, populations of Gag-specific IFN- $\gamma$ -producing CD8<sup>+</sup> T cells in the prime-boost group averaged 0.32%, and populations of CD4<sup>+</sup> T cells averaged 0.11%. Three days after viral challenge, the average for Ag-specific IFN- $\gamma$ -producing CD8<sup>+</sup> T cells rose to 0.61%, and that for CD4<sup>+</sup> T cells to 0.38%. Gag-specific IFN- $\gamma$ -producing CD8<sup>+</sup> T cells averaged 0.18% for the DNA group and 0.25% for the rDIs group on the day of challenge, with those averages rising to 0.28 and 0.39%, respectively, by 3 days after challenge. Furthermore, the averages for Gag-specific CD4<sup>+</sup> T cells in the DNA and rDIs groups rose from 0.08 and 0.10 to 0.14 and 0.23%, respectively. The number of Ag-specific IFN- $\gamma$ -pro-

ducing CD8<sup>+</sup> and CD4<sup>+</sup> T cells in the control group was not affected by viral challenge. Thus, compared with the other three groups of animals, the prime-boost group showed the most significant enhancement of Ag-specific cellular immune responses after viral challenge, suggesting that Gag-specific memory T cell responses may be efficiently generated in animals by immunization with the prime-boost vaccine regimen.

To test the kinetics of humoral immune responses after SHIV challenge, we measured serum IgG titers to SIV Gag and SHIV 89.6P Env in all animals of each group (Fig. 4, A and B). The SIV Gag-specific IgG titers in all vaccinated animals were rapidly elevated and reached peak levels within 4 wk after challenge (Fig. 4A). The peak IgG titers in the prime-boost, DNA, and rDIs groups averaged  $14,520 \pm 2,508$ ,  $5,240 \pm 1,099$ , and  $8,400 \pm 1,114$ ,

**FIGURE 3.** Flow cytometric analysis of SIV Gag-specific IFN- $\gamma$ -producing CD8<sup>+</sup> T cells. One week after rDIs boost immunization, freshly isolated PBMC were stimulated with 0.2  $\mu$ M pooled SIV Gag peptides and stained for anti-CD3, -CD8, and -IFN- $\gamma$ . Twenty thousand lymphocyte-gated events were acquired. Upper and lower quadrants represent the frequencies of CD8<sup>+</sup> IFN- $\gamma$ <sup>+</sup> and CD4<sup>+</sup> IFN- $\gamma$ <sup>+</sup> T cells, respectively. Numbers indicate the percentage of cells in each quadrant.



respectively, with the increase in the prime-boost group reaching statistical significance ( $p < 0.01$ ), compared with that in the DNA and rDIs groups. The Env-specific IgG appeared by 4 wk after challenge and reached peak levels between 7 and 11 wk. The peak IgG titers in the prime-boost, DNA, and rDIs groups averaged  $5,200 \pm 1,839$ ,  $3,180 \pm 701$ , and  $4,533 \pm 833$ , respectively. Both the SIV Gag- and Env-specific IgG titers in the three vaccinated groups maintained high levels and persisted throughout the challenge period. In contrast, no IgG response to Gag and Env was detected in the control group. High titers of Env-specific IgG, but only very low levels of neutralization Ab responses to SHIV-C2/1, were induced in the DNA- and rDI-vaccinated groups (Fig. 4C). In contrast, the prime-boost macaques, especially M1, had high levels of neutralization Ab responses (viral neutralization  $>70\%$ ). Thus, these results show that the prime-boost vaccine with DNA/rDIs predominantly elicits SIV Gag-specific humoral responses in immunized animals and generates SHIV Env-specific binding and neutralization Abs after challenge with SHIV.

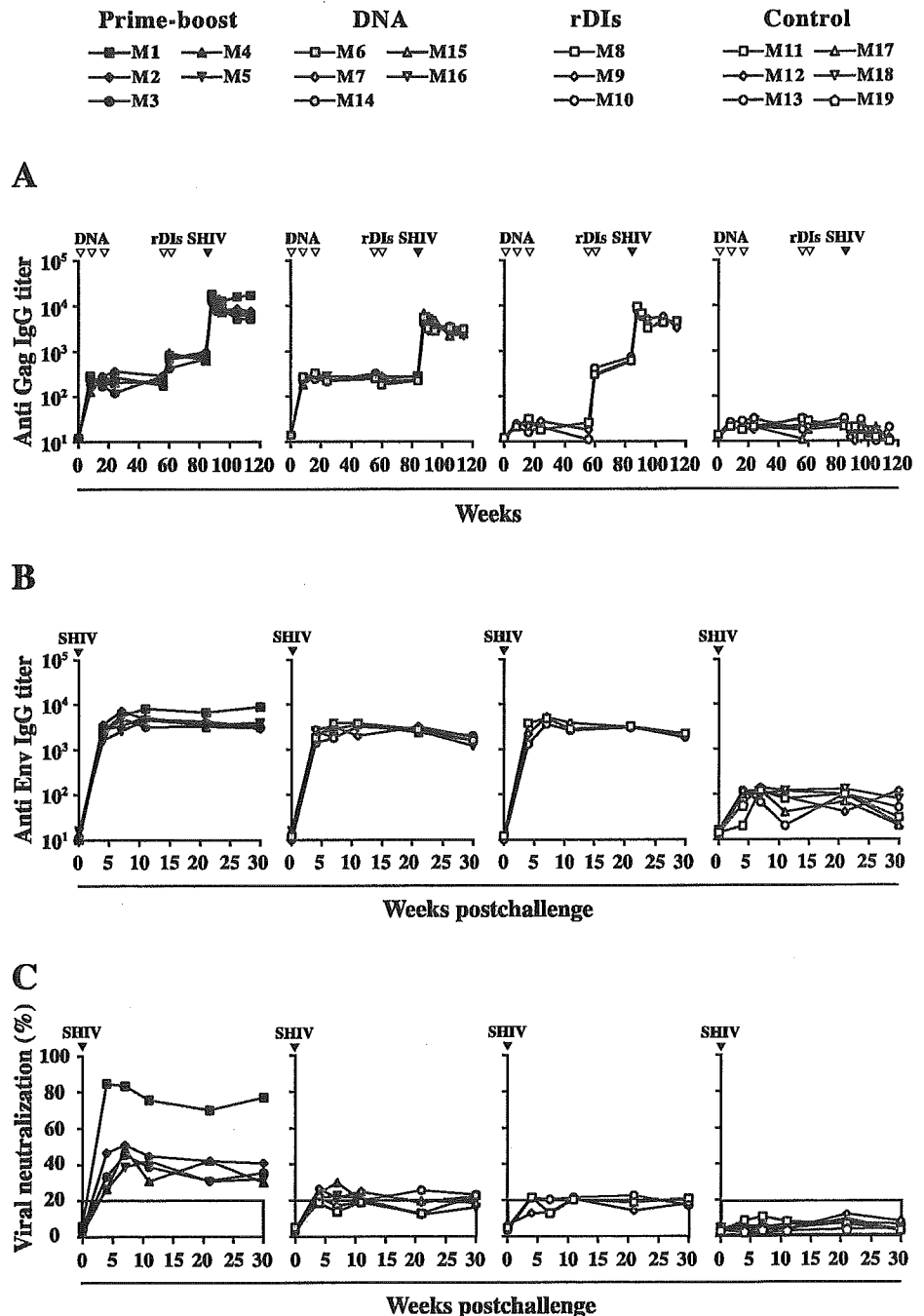
#### *Macaques of the prime-boost group control plasma viral load and block CD4<sup>+</sup>-T cell depletion*

As noted above, the five macaques in the prime-boost group developed Ag-specific positive immunity after viral challenge. In these ma-

caques, plasma viral loads were most attenuated and CD4<sup>+</sup> T cell counts best maintained in peripheral blood (Fig. 6). Peak viral loads occurred 2 wk after challenge in each group. The geometric means of the viral RNA copies were  $1.1 \times 10^7$  copies in the prime-boost group,  $4.7 \times 10^7$  copies in the DNA group,  $4.1 \times 10^7$  copies in the rDIs group, and  $4.5 \times 10^7$  copies in the control group (Fig. 6A). The difference observed in geometric mean peak viremia for the prime-boost and rDIs groups was significant ( $p < 0.05$ ). Levels of peak viremia in the rDIs and control groups did not significantly differ. The peak viral loads in each had decreased by 7 wk after challenge, and the geometric means of the viral RNA copies from 7 to 30 wk were  $8.1 \times 10^3$  copies (ranging from  $7.1 \times 10^3$  to  $9.4 \times 10^3$  copies) in the prime-boost group,  $1.1 \times 10^6$  copies (ranging from  $2.5 \times 10^5$  to  $6.6 \times 10^6$  copies) in the DNA group,  $7 \times 10^4$  copies (ranging from  $5.3 \times 10^4$  to  $1.1 \times 10^5$  copies) in the rDIs group, and  $6.8 \times 10^6$  copies (ranging from  $2.0 \times 10^6$  to  $5.2 \times 10^7$  copies) in the control group (Fig. 6A). From 7 to 30 wk, the differences in the geometric means of the viral RNA copies between prime-boost and DNA groups ( $p < 0.01$ ), prime-boost and rDIs groups ( $p < 0.01$ ), and DNA and rDIs groups ( $p < 0.01$ ) vs DNA and control groups ( $p < 0.05$ ) were significant.

Two weeks after challenge, both DNA and control groups showed a serious depletion of CD4<sup>+</sup> T cells (to  $<50$  cells) and a corresponding increase in viral RNA. In contrast, the prime-boost



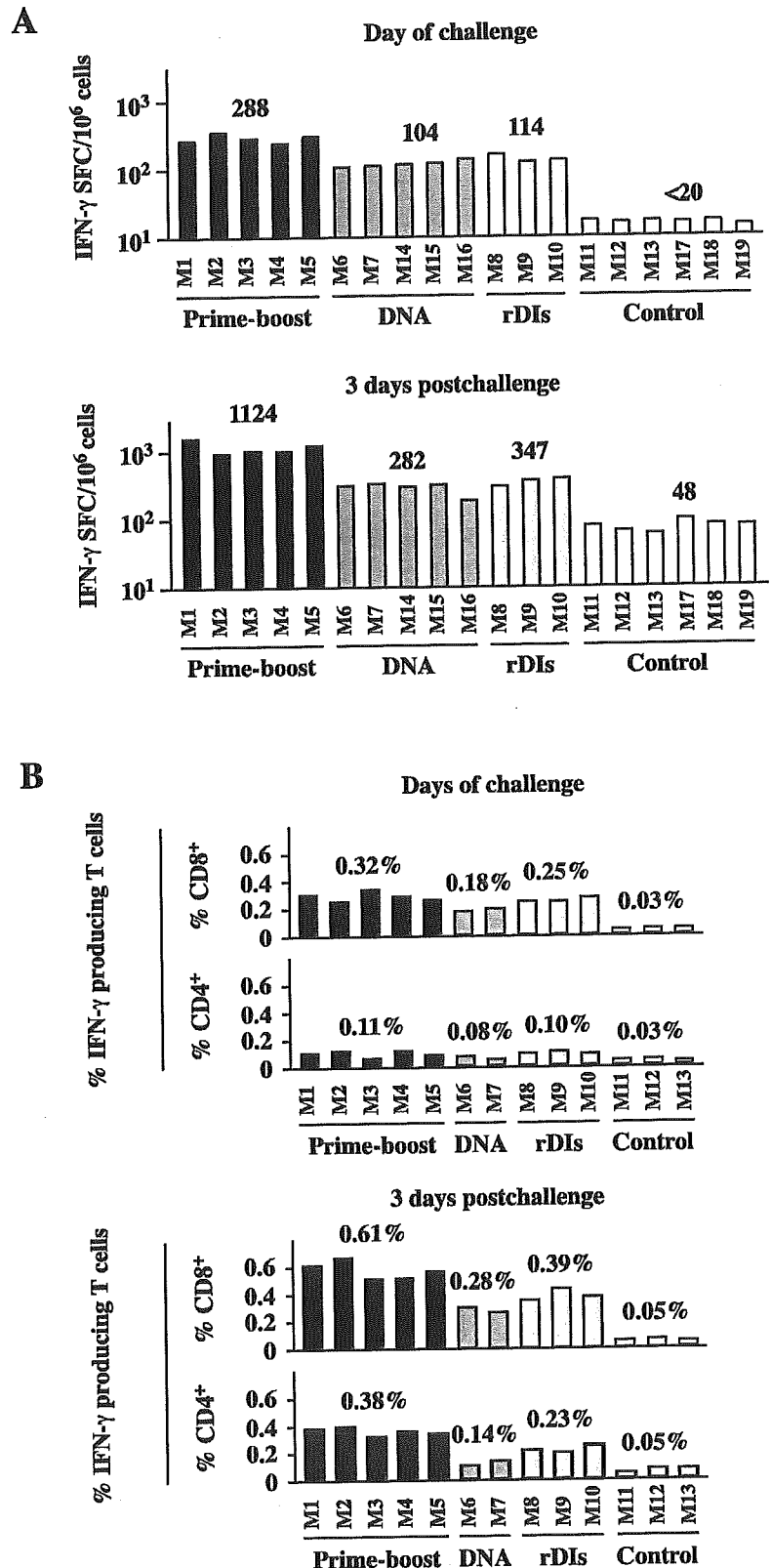


**FIGURE 4.** Kinetics of serum IgG titers specific to SIV Gag and SHIV 89.6P Env. *A*, SIV p27 Gag- and Env-specific IgG titers after immunization and after challenge. *B*, SHIV 89.6P Env-specific IgG titers. *C*, SHIV-specific neutralization responses. End-point titers of Gag- and Env-specific serum IgG and the percentage of SHIV-specific neutralization responses were measured at each time point. Results represent the average titer and percentage of the average viral neutralization value.

group maintained its CD4<sup>+</sup> T cell counts up to 30 wk after challenge (Fig. 6*B*). Four of the five macaques (M2–5) in the prime-boost group exhibited a gradual decrease in CD4<sup>+</sup> T cell counts; however, the macaques maintained an average of 254–303 cells from 2 to 30 wk after challenge. The one remaining macaque in the group (M1) maintained an average of 833 CD4<sup>+</sup> T cells (ranging from 630 to 1230 cells) and exhibited levels of viral RNA (<500 copies) that were undetectable except when peak viremia was reached at 2 wk ( $5.7 \times 10^7$  copies) and transient viral replication occurred at 7 wk ( $1.5 \times 10^4$  copies; Fig. 6, *A* and *B*).

To characterize the changes in the CD4<sup>+</sup> T cell subset in peripheral blood of each group after SHIV challenge, we used flow cytometric analysis to obtain an absolute count and to distinguish among the CD29<sup>+</sup>, CD45<sup>+</sup>, and CD28<sup>+</sup> cell subpopulations (Fig.

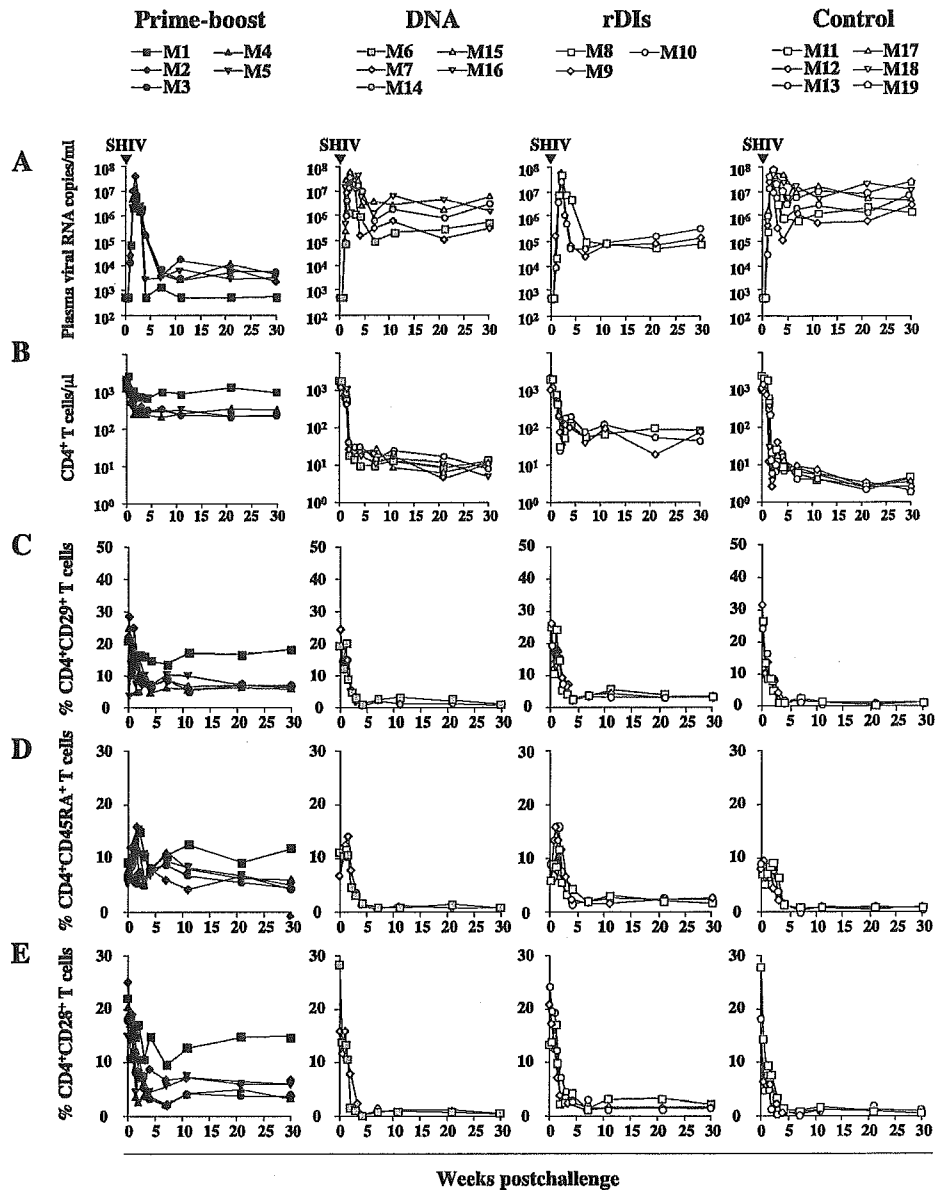
6, *C–E*). By 2 wk after challenge, a sharp decrease in the CD29<sup>+</sup> subset of CD4<sup>+</sup> T cells was seen in the DNA, rDIs and control groups (Fig. 6*C*). From 2 to 30 wk after challenge, the average number of this subset of cells in the DNA, rDIs, and control groups was 1.21% (ranging from 0.79 to 2.01%), 2.14% (ranging from 1.80 to 3.59%), and 1.03% (ranging from 0.55 to 1.89%), respectively. Similarly, the CD45RA<sup>+</sup> subset of CD4<sup>+</sup> T cells in the three groups rapidly declined by 2 wk after challenge, with the average of naive cells from 2 to 30 wk being 1.04% (ranging from 0.72 to 1.32%) in the DNA group, 2.83% (ranging from 1.04 to 4.78%) in the rDIs group, and 0.88% (ranging from 0.34 to 1.34%) in the control group (Fig. 6*D*). In contrast, the prime-boost group maintained the highest frequencies of both the CD29<sup>+</sup> subset, ranging from 8.0 to 9.63% with an average of 8.82% (Fig. 6*C*), and



**FIGURE 5.** Comparison of IFN- $\gamma$  ELISPOT activity and intracellular IFN- $\gamma$ -producing T cells specific for SIV Gag in PBMC before and after viral challenge. *A*, ELISPOT activity. The numbers above the data bars represent the geometric means of SFC levels in each group. *B*, Intracellular IFN- $\gamma$ -producing T cells. On the day of SHIV challenge and 3 days after SHIV challenge, freshly isolated cells were stimulated with SIV Gag peptides and stained for CD3, CD8, and IFN- $\gamma$ . Numbers represent the percent average of the CD4<sup>+</sup> and CD8<sup>+</sup> T cell frequencies.

the CD45<sup>+</sup> subset, ranging from 6.29 to 9.16% with an average of 7.59% (Fig. 6, *C* and *D*). Flow cytometric analyses also revealed that the number of CD4<sup>+</sup> T cells expressing the costimulatory molecule CD28 rapidly dropped in the DNA, rDIs, and control groups by 2 wk after challenge (Fig. 6*E*). The average of

CD4<sup>+</sup>CD28<sup>+</sup> T cells from 2 to 30 wk after challenge in the DNA, rDIs, and control groups was 0.74% (ranging from 0.23 to 1.12%), 1.66% (ranging from 1.01 to 2.6%), and 0.91% (ranging from 0.61 to 1.13%), respectively. In contrast, CD28<sup>+</sup>CD4<sup>+</sup> T cells in the prime-boost group ranged from 5.27 to 7.26%, with an average of



**FIGURE 6.** Kinetics of viral loads, CD4<sup>+</sup> T cell counts, and subpopulations of CD4<sup>+</sup> T cells in experimental groups after SHIV challenge. *A*, Plasma viral loads. Plasma viral loads were measured using the real-time PCR system. Levels <500 copies/ml were considered undetectable in this system. *B*, CD4<sup>+</sup> T cell counts. Whole blood was stained for CD3, CD4, and CD8 Abs, and CD4<sup>+</sup> T cell counts were determined using flow cytometry. *C*, CD4<sup>+</sup>CD29<sup>+</sup> T cells. *D*, CD4<sup>+</sup>CD45RA<sup>+</sup> T cells. *E*, CD4<sup>+</sup>CD28<sup>+</sup> T cells. CD4<sup>+</sup> T cell subpopulations were not reduced in the prime-boost animal group.

6.75%. Thus, the prime-boost group maintained CD29<sup>+</sup>, CD45RA<sup>+</sup>, and CD28<sup>+</sup> cell subpopulations in CD4<sup>+</sup> T cells after viral challenge.

*Controls of viremia and stability of CD4<sup>+</sup> blood lymphocytes correlate with Gag-specific IFN-γ SFC and neutralization Ab responses*

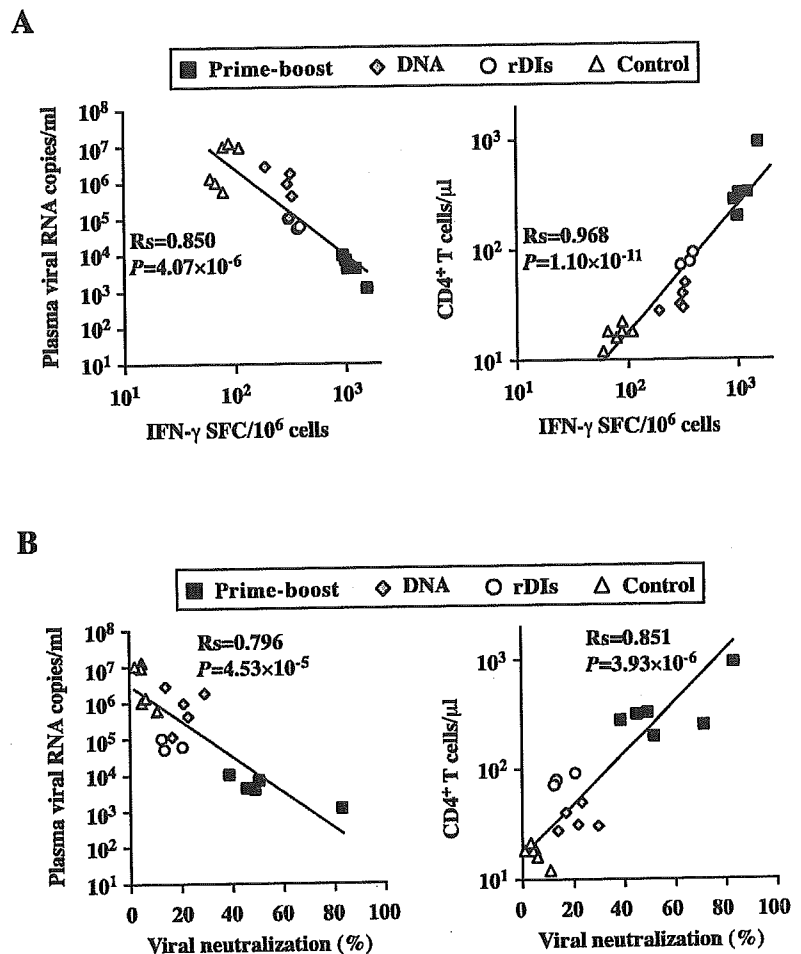
Because positive immune responses were detected in the animals immunized with the prime-boost vaccine of DNA/vaccinia DIs, we examined whether any immune responses correlated with the positive immunities using Spearman's rank correlation test (Fig. 7). The set-point levels of plasma viral RNA and CD4<sup>+</sup> T cell counts 7 wk after challenge significantly correlated with the Gag-specific IFN-γ SFC levels 3 days after challenge (plasma viral RNA levels vs Gag-specific IFN-γ SFC levels:  $R_s = 0.850, p = 4.07 \times 10^{-6}$ ; CD4<sup>+</sup> T cell counts vs Gag-specific IFN-γ SFC levels:  $R_s = 0.968, p = 1.10 \times 10^{-11}$ ; Fig. 7A). Interestingly, there was less correlation between the same set-point plasma viral RNA levels and CD4<sup>+</sup> T cell counts and the neutralization Ab responses 7 wk

after challenge (plasma viral RNA levels vs percent viral neutralization:  $R_s = 0.796, p = 4.53 \times 10^{-5}$ ; CD4<sup>+</sup> T cell counts vs percent viral neutralization:  $R_s = 0.851, p = 3.93 \times 10^{-6}$ ). No correlation at all was observed between positive immune responses and anti-Gag and anti-Env Ab titers (data not shown).

**Discussion**

It is believed likely that HIV-specific immune responses are associated with a decline in viral load and CD4<sup>+</sup> T cell maintenance. Our current study using the macaque model suggests that the prime-boost regimen, that is, priming with SIVgag/pol DNA followed by boosting with rDIsSIVgag/pol, modifies pathogenic SHIV infection. Furthermore, when the relationship between protection and the levels of immune responses was analyzed, we found that Gag-specific IFN-γ T cells showed a strong correlation and neutralization responses a weaker correlation with the suppression of plasma viral RNA levels and maintenance of CD4<sup>+</sup> T cell counts. These results accord with previous reports associating

**FIGURE 7.** Correlations between protection and immune responses. **A**, The correlations between the decline in plasma viral RNA and the increased number of Gag-specific IFN- $\gamma$  SFC, and between CD4<sup>+</sup> T cell counts and Gag-specific IFN- $\gamma$  SFC. **B**, Correlations between the decline in plasma viral RNA and increased neutralization Ab responses, and between CD4<sup>+</sup> T cell counts and neutralization Ab responses. Correlations are calculated using Spearman's rank correlation test.



viral control with cellular immune responses in animals immunized with a prime-boost vaccination of either DNA/MVA (11) or cytokine-augmented DNA (36) encoding *gag* and *env* genes, followed by SHIV challenge. Neutralization Ab production was also detected in the animals (11, 36). Our new observations in vaccine research include the following: 1) Because positive immune responses better correlated with T cell than neutralization responses, it is probable that control of the plasma viral load and CD4<sup>+</sup> cell counts was achieved by virus-specific cellular immune responses. 2) Although our vaccine target was only Gag in this strategy, neutralization titers were detected in the prime-boost group that were higher than those induced in animals immunized with DNA, rDIs, or vector controls alone. These higher titers of the neutralization Ab responses against challenge virus might account for the presence of a high number of CD4<sup>+</sup> T cells in prime-boost animals (Fig. 6B) and might be associated with the production of neutralization Ab. It may, therefore, be reasonable to conclude that anti-Env neutralization Abs were effectively induced in the animals after SHIV challenge. Thus, we suggest that not only cellular responses, but also neutralizing Ab responses, elicited by the challenge virus may play a role in the pathogenesis of HIV/AIDS in the macaque model. 3) This vaccination regimen consisted of DNA and a nonreplicating vaccinia virus DIs, which is very safe even in immunodeficient states. Although other highly attenuated vaccinia strains replicate under synchronized viral infections to mammalian cells (37, 38), the DIs does not replicate in any mammalian cells tested because of natural big deletion of the genome (22, 23, 39). Thus, DIs vaccination eliminates the risk of a disseminated or pro-

gressive vaccinia viral infection in the immunocompromised, HIV-infected individual. Therefore, the DNA/DIs vaccine will be most safe in mammals and may be suitable for therapeutic vaccine.

Recently, we demonstrated that priming with SIV*gag/pol* DNA, followed by boosting with rDIsSIV*gag/pol* generated both Th1-type CD8<sup>+</sup> and CD4<sup>+</sup> T cell responses specific for SIV Gag, resulting in the protection of immunized mice from a wild-type vaccinia virus recombinant expressing SIV Gag and Pol (23). Our previous mouse and macaque results (23, 40) (Fig. 6) showed that DNA alone was not as effective at inducing positive immunity in the macaque AIDS model as had been reported by others (11, 36). This discrepancy may depend on differences in DNA preparation, for example, whether the target HIV DNA was optimized to the human codon.

Although the exact immune mechanism responsible for protection from viral infection is not yet fully understood, both Ag-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses were clearly enhanced by viral infection in the prime-boost-immunized animals that exhibited a pronounced attenuation of plasma viral load. Our finding that challenge with the highly pathogenic SHIV virus enhances cellular immunity confirms the results of a recent study (41). It has been demonstrated that HIV-specific CD8<sup>+</sup> T cell responses play an important role in controlling viral replication by cytolysis and cytokine and/or anti-virus factor production (1, 2, 3, 42, 43). Others have also documented that HIV-specific CD4<sup>+</sup> T cell responses contribute to virus control or the slowing of disease progression (44–46). The critical role played by CD4<sup>+</sup> T cell responses against viral infections was also reported in studies of murine