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Induction of Positive Cellular and Humoral Immune Responses by a Prime-Boost Vaccine Encoded with Simian Immunodeficiency Virus gag/pol

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

It is believed likely that immune responses are responsible for controlling viral load and infection. In this study, macaques were primed with plasmid DNA encoding SIV gag and pol genes (SIVgag/pol DNA) and then boosted with replication-deficient vaccinia virus DIs recombinant expressing the same genes (rDIsSIVgag/pol), this prime-boost regimen generated higher levels of Gag-specific CD4+ and CD8+ T cell responses than did either SIVgag/pol DNA or rDIsSIVgag/pol alone. When the macaques were i.v. challenged with pathogenic simian/HIV, the prime-boost group maintained high CD4+ T cell counts and reduced plasma viral loads up to 30 wk after viral challenge, whereas the rDIsSIVgag/pol group showed only a partial attenuation of the viral infection, and the group immunized with SIVgag/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+ 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)3 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+ 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 SIVgag/pol DNA and rDIsSIVgag/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+ and CD8+ T cell immune responses than those vaccinated with either DNA or rDIs alone. When such mice were challenged with SIV gag/pol expressing

3 Abbreviations used in this paper: MVA, modified vaccinia virus Ankara; rDIsSIVgag/pol, recombinant DIs expressing SIVgag and pol; SFC, spot-forming cell; SHIV, simian-human immunodeficiency virus; SIVgag/pol DNA, plasmid DNA encoding SIV gag and pol genes; TCID50, 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 (SIVgag/pol DNA) and recombinant DIs expressing the same genes (rDIsSIVgag/pol) were prepared as previously described (22, 23, 29). pcDN3.1 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

Approximately 10^6 of fresh PBMC were incubated with 0.2 μg/ml purified macaque IgG was incubated with 100 50% tissue culture infectious doses (TCID50) 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 SHIV 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 × 10^4 RNA copies) were subjected to RT-PCR using an SIVmac239–1224 forward, SIVmac239–1326 reverse primer pair and an FAM-SIV-1227T 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^+ T cells

Approximately 10^6 fresh PBMC were stained with anti-human CD4 (clone SK3; 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 μl of Flow Count (Beckman Coulter) was added. A FACSCalibur flow cytometer (BD Biosciences) was used to acquire 20,000 lymphocyte-gated events, which were then analyzed with CellQuest software.

Statistical analysis

Data are expressed as the mean ± 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 virus 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 rDIsSIVgag/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 SIVgag/pol DNA at 8-wk intervals, followed by two injections of 10^6 PFU of rDIsSIVgag/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 SIVgag/pol DNA at
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 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_{50} 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-γ 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-γ SFC than did a single immunization in the prime-booster and DNA groups (p < 0.05; Fig. 2, A and B). The numbers of IFN-γ-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 rDIsSIVgag/pol (rDIs group) generated higher IFN-γ 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-γ SFC detected in the control group. Collectively, our findings show that the DNA/rDIs prime-booster immunization efficiently induced immunodeficiency virus-specific ELISPOT activity in macaques.

To substantiate the induction of cellular immunity specific for SIV Gag, intracellular IFN-γ 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-γ-producing CD4^+ and CD8^+ T cells. The frequencies of Gag-specific IFN-γ-producing CD8^+ and CD4^+ 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-γ-producing CD8^+ and CD4^+ T cells immunized with either SIVgag/pol DNA (average of CD8^+ T cells, 0.095%; average of CD4^+ T cells, 0.015%) or rDIsSIVgag/pol (average of CD8^+ T cells, 0.27%; average of CD4^+ 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-γ 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-γ 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-γ-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.
Intracellular IFN-γ staining of CD8+ and CD4+ 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-γ-producing CD8+ T cells in the prime-boost group averaged 0.32%, and populations of CD4+ T cells averaged 0.11%. Three days after viral challenge, the average for Ag-specific IFN-γ-producing CD8+ T cells rose to 0.61%, and that for CD4+ T cells to 0.38%. Gag-specific IFN-γ-producing CD8+ 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+ T cells in the DNA and rDIs groups rose from 0.08 to 0.10 to 0.14 and 0.23%, respectively. The number of Ag-specific IFN-γ-producing CD8+ and CD4+ 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 ± 2,508, 5,240 ± 1,099, and 8,400 ± 1,114,
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 ± 1,839, 3,180 ± 701, and 4,533 ± 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 to SHIV (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.

Macaque of the prime-boost group control plasma viral load and block CD4⁺ T-cell depletion

As noted above, the five macaques in the prime-boost group developed Ag-specific positive immunity after viral challenge. In these macaques, plasma viral loads were most attenuated and CD4⁺ 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 × 10⁷ copies in the prime-boost group, 4.7 × 10⁶ copies in the DNA group, 4.1 × 10⁷ copies in the rDIs group, and 4.5 × 10⁷ 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 × 10³ copies (ranging from 7.1 × 10³ to 9.4 × 10³ copies) in the prime-boost group, 1.1 × 10⁵ copies (ranging from 2.5 × 10⁵ to 6.6 × 10⁵ copies) in the DNA group, 7 × 10⁶ copies (ranging from 5.3 × 10⁶ to 1.1 × 10⁷ copies) in the rDIs group, and 6.8 × 10⁶ copies (ranging from 2.0 × 10⁶ to 5.2 × 10⁶ 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⁺ T cells (to <50 cells) and a corresponding increase in viral RNA. In contrast, the prime-boost...
group maintained its CD4⁺ T cell counts up to 30 wk after challenge (Fig. 6B). Four of the five macaques (M2–5) in the prime-boost group exhibited a gradual decrease in CD4⁺ 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⁺ 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 × 10⁷ copies) and transient viral replication occurred at 7 wk (1.5 × 10⁷ copies; Fig. 6B).

To characterize the changes in the CD4⁺ 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⁺, CD45⁺, and CD28⁺ cell subpopulations (Fig. 6, C–E). By 2 wk after challenge, a sharp decrease in the CD29⁺ subset of CD4⁺ T cells was seen in the DNA, rDIs, and control groups (Fig. 6C). 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⁺ subset of CD4⁺ 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. 6D). In contrast, the prime-boost group maintained the highest frequencies of both the CD29⁺ subset, ranging from 8.0 to 9.63% with an average of 8.82% (Fig. 6C), and
the CD45^+ 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^+ T cells expressing the costimulatory molecule CD28 rapidly dropped in the DNA, rDIs, and control groups by 2 wk after challenge (Fig. 6E). The average of CD4^+CD28^+ 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^-CD4^+ T cells in the prime-boost group ranged from 5.27 to 7.26%, with an average of

FIGURE 5. Comparison of IFN-γ ELISPOT activity and intracellular IFN-γ-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-γ-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-γ. Numbers represent the percent average of the CD4^- and CD8^- T cell frequencies.
6.75%. Thus, the prime-boost group maintained CD29^+,
CD45RA^+ and CD28^+ cell subpopulations in CD4^+ T cells after
viral challenge.

Controls of viremia and stability of CD4^+ 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 pos-
itive immunities using Spearman’s rank correlation test (Fig. 7).
The set-point levels of plasma viral RNA and CD4^+ 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: Rs = 0.850, p = 4.07 × 10^-6; CD4^+ T cell counts vs Gag-specific IFN-γ SFC levels: Rs = 0.968, p = 1.10 × 10^-11; Fig. 7A). Interestingly, there was less
correlation between the same set-point plasma viral RNA levels
and CD4^+ T cell counts and the neutralization Ab responses 7 wk
after challenge (plasma viral RNA levels vs percent viral neutral-
ization: Rs = 0.796, p = 4.53 × 10^-5; CD4^+ T cell counts vs percent viral neutralization: Rs = 0.851, p = 3.93 × 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 asso-
ciated with a decline in viral load and CD4^+ T cell maintenance.
Our current study using the macaque model suggests that the
prime-boost regimen, that is, priming with SIV^gag/pol DNA fol-
lowed by boosting with rDIsSIV^gag/pol, modifies pathogenic
SHIV infection. Furthermore, when the relationship between pro-
tection 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 sup-
pression of plasma viral RNA levels and maintenance of CD4^+ T
cell counts. These results accord with previous reports associating

FIGURE 6. Kinetics of viral loads, CD4^+ T cell counts, and sub-
populations of CD4^+ T cells in experimental groups after SHIV chal-
lenge. A, Plasma viral loads. Plasma viral loads were measured using the
real-time PCR system. Levels <500 copies/ml were considered undetect-
able in this system. B, CD4^+ T cell counts. Whole blood was stained for
CD3, CD4, and CD8 Abs, and CD4^+ T cell counts were determined using
flow cytometry. C, CD4^+CD29^+ T cells. D, CD4^+CD45RA^+ T cells. E,
CD4^+CD28^+ T cells. CD4^+ T cell subpopulations were not reduced in
the prime-boost animal group.
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\(^+\) 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\(^+\) T cells in prime-boost animals (Fig. 6B) and might be associated with the production of neutralization Abs. 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 progressive 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\(^+\) and CD4\(^+\) 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\(^+\) and CD8\(^+\) 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 the highly pathogenic SHIV virus enhances cellular immunity confirms the results of a recent study (41). It has been demonstrated that HIV-specific CD8\(^+\) T cell responses play an important role in controlling viral replication by cytolyis and cytokine and/or anti-virus factor production (1, 2, 3, 42, 43). Others have also documented that HIV-specific CD4\(^+\) T cell responses contribute to virus control or the slowing of disease progression (44–46). The critical role played by CD4\(^+\) T cell responses against viral infections was also reported in studies of murine

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\(^+\) 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\(^+\) T cell counts and neutralization Ab responses. Correlations are calculated using Spearman’s rank correlation test.
lymphatic choriomeningitis viral infection (47) and CMV immunity in bone marrow transplant recipients (48). DNA/poxvirus prime-boost vaccination induced a high frequency and a high avidity of CD8+ cytotoxic T lymphocyte populations (49), with the magnitude of HIV/SIV-specific CD4+ and/or CD8+ T lymphocyte responses in the course of infection inversely correlating with the viral load (50, 51). In addition, MHC class I molecules loading CTL epitopes may help control viral replication (52–56). The exact mechanisms underlying protective immune responses against HIV-1 remain a subject of debate; however, the above studies suggest that the simultaneous induction after vaccination of both Ag specific CD8+ and CD4+ T cell responses may make it possible to attenuate immunodeficient viral infection. In this study we showed the efficacy of the prime-boost vaccination by monitoring IFN-γ ELISPOT, intracellular IFN-γ, and Ab responses. In the prime-boost group, boosting with rDIsSIVgag/pol induced ELISPOT responses (average of 1209 SFC) almost 10-fold higher than those induced by SIVgag/pol DNA (average of 154 SFC). In addition, intracellular IFN-γ-staining revealed that the prime-boost vaccination generated high levels of Gag-specific intracellular IFN-γ-producing CD8+ T cells (average, 0.82%; range, 0.51–1.22%) as well. However, lower Gag-specific T cell responses were observed in macaques vaccinated with either SIVgag/pol DNA or rDIsSIVgag/pol alone than with the prime-boost regimen. In contrast to the strong Gag-specific T cell responses generated by the prime-boost vaccination, humoral responses specific for the same Ag were apparently low throughout the course of immunization. Although the peak IgG titers in the prime-boost group were observed after the first or second boosting with rDIsSIVgag/pol, Ab titers remained low. These results are in line with our previous study using the mouse model (23), suggesting that our prime-boost vaccine immunodominantly generates SIV Gag-specific cellular responses in macaques.

Monitoring ELISPOT and intracellular IFN-γ T cell responses specific for Gag revealed that responses decreased at the time of challenge with pathogenic SHIV, but then rapidly recovered. Gag-specific IFN-γ ELISPOT responses in the prime-boost group averaged 288 SFC on the day of challenge and increased to 1124 SFC on day 3 after challenge. The population of intracellular IFN-γ-producing CD8+ and CD4+ T cells specific for Gag also increased from an average of 0.32 to 0.61% and from an average of 0.11 to 0.38%, respectively, suggesting that our prime-boost vaccine generated a high frequency of very responsive CD4+ and CD8+ memory T cells that immediately reactivated sufficient levels of the Ag-specific immune responses against the SHIV Ag. Furthermore, a kinetic study of plasma viral loads and counts of CD4+ T cells after challenge with SHIV revealed different patterns for each group. Although peak plasma viral loads were observed 2 wk after challenge in all groups, the number of plasma RNA copies peaking at that time in the prime-boost group were ~5-fold lower than in other groups, with numbers remaining depressed during the period extending from 7 to 30 wk after infection. However, high CD4+ T cell counts were maintained in the prime-boost group. These results suggest a correlation between both plasma viral loads and the maintenance of high CD4+ T cell counts and T cell response levels.

With regard to safety of vaccinia DIs as a vaccine vector, its viral replication occurs only in chick embryo fibroblasts, not in any mammalian cell lines tested (22, 24–26, 57). Because a vaccine regimen combining DNA and a defective DIs vector would not run the risk that the virus used as vector might replicate and disseminate, it would pose less of a risk to a severely immunocompromised host. Furthermore, in this study using the macaque model, we demonstrated that the cellular immune responses generated by the prime-boost vaccination were higher than those induced by vaccination with either DNA or rDIs alone and that response levels correlated to plasma viral loads and CD4+ T cell counts after challenge with pathogenic SHIV. In summary, these results demonstrate that the new prime-boost regimen safely and effectively elicits anti-immunodeficiency viral immunity, suggesting its promise as a potential vaccine against HIV-1 infection as well as against HIV-induced disease progression.

Current macaque models of HIV, SIV, and SHIV may fall short of precisely mirroring human HIV infection. In some macaque HIV/AIDS models, SIVmac239 has been targeted as a desirable challenge virus, because it is a typical CCR5-tropic SIVmac and can cause both chronic and progressive disease in macaques (41, 58, 59). However, the virus is very difficult to neutralize and also very difficult to clear even from animals that have been previously immunized with homologous recombinant vector-based vaccines (41, 58, 59). Only live attenuated SIV has been reported to control SIVmac239 (T. Allen, Global HIV Vaccine Enterprise Meeting, Washington, October 21, 2004). Although there may be no macaque model suitable for evaluating the efficacy of an SIV or HIV experimental immunogen, in this study we clearly showed that vaccination with an SIV experimental immunogen consisting of SIVgag/pol DNA and replication-defective rDIsSIVgag/pol caused a pronounced attenuation of the infection caused by a highly pathogenic variant of SHIV-C2/1 in all five macaques tested. SHIV-C2/1, used as challenge virus, is a variant of SHIV89.6. Because SHIV89.6 does not induce both a marked decline in CD4+ cells and a high level of plasma viral load in cynomolgus macaques, we passaged serum from virus-infected cynomolgus macaques. The variant was obtained by the serum passages using cynomolgus macaques inoculated with SHIV89.6, and it induced high levels of viremia (1–10 × 10⁷ viral RNA copies/ml) and marked CD4+ T cell depletion (<10/µl) within 2 and 3 wk after viral inoculation (30, 31, 39). Furthermore, genomic study revealed 16 mutations of genomic DNA and 15 amino acid mutations in the Env region of parental virus. Thus, the cynomolgus AIDS model challenged with SHIV-C2/1 may represent primary HIV-1 infection in humans. These results should prove useful in determining how potent the new prime-boost vaccine regimen might be at eliciting anti-immunodeficiency virus immunity.

HIV-1 has been reported to preferentially infect CD45RO+CD4+ T cells in the early stages of infection, with the number of CD45RA−CD4+ T cells declining in later stages (60–62). Furthermore, the loss of this subpopulation of CD4+ T cells during the early phase of immunodeficiency virus infection correlates to disease progression (63, 64), whereas the low CD45RA−CD4+ T cell levels in the late stages of infection correlate with an increased risk of death (65–67). The levels of CD4+ T cells expressing the CD28 molecule have also been demonstrated to correlate with disease progression (68, 69). To confirm the effect of prime-boost immunity after SHIV challenge, we analyzed the kinetics of CD4+ T cells expressing CD29+, CD45RA+, and CD28+ molecules. We observed that the prime-boost group maintained the subpopulations of CD4+ T cells throughout the course of infection, with an average of 8.82% CD29+ cells, 7.59% CD45RA− cells, and 6.75% CD28+ cells. In contrast, CD4+ T cell populations in the other DNA and rDIs groups were reduced to <3%. These results suggest that immunization with the new prime-boost regimen induces protective immunity while maintaining the levels of the various CD4+ T cell subpopulations.

In summary, our study has shown that the vaccine strategy that primes with DNA and then boosts with the replication-defective
vaccinia virus DNA vaccines can elicit both CD4+ and CD8+ T cell responses specific for SIV Gag, resulting in protection of the immunized macaques from pathogenic SHIV. However, it remains to be elucidated whether the gag/pol-encoding vaccine may elicit a protective effect against various viral challenges, such as CCR5-tropic viruses and other primary viruses. Nonetheless, this new regimen’s twin merits of safety and efficacy position it as a promising vaccine candidate against HIV-1 infection as well as against HIV-induced disease progression.

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Disclosures

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