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Prior DNA immunization enhances immune response to dominant and subdominant viral epitopes induced by a fowlpox-based SIVmac vaccine in long-term slow-progressor macaques infected with SIVmac251

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Abstract

A therapeutic vaccine for individuals infected with HIV-1 and treated with antiretroviral therapy (ART) should be able to replenish virus-specific CD4+ T-cells and broaden the virus-specific CD8+ T-cell response in order to maintain CD8+ T-cell function and minimize viral immune escape after ART cessation. Because a combination of DNA and recombinant poxvirus vaccine modalities induces high levels of virus-specific CD4+ T-cell response and broadens the cytolytic activity in naive macaques, we investigated whether the same results could be obtained in SIVmac251-infected macaques. The macaques studied here were long-term nonprogressors that naturally contained viremia but were nevertheless treated with a combination of antiviral drugs to assess more carefully the effect of vaccination in the context of ART. The combination of a DNA expressing the *gag* and *pol* genes (DNA-SIV-*gp*) of SIVmac239 followed by a recombinant fowlpox expressing the same SIVmac genes (FP-SIV-*gp*) was significantly more immunogenic than two immunizations of FP-SIV-*gp* in SIVmac251-infected macaques treated with ART. The DNA/FP combination significantly expanded and broadened Gag-specific T-cell responses measured by tetramer staining, ELISPOT, and intracellular cytokine staining and measurement of ex vivo cytolytic function. Importantly, the combination of these vaccine modalities also induced a sizeable expansion in most macaques of Gag-specific CD8-(CD4+) T-cells able to produce TNF-α. Hopefully, this modality of vaccine combination may be useful in the clinical management of HIV-1-infected individuals.

Keywords: Vaccination; AIDS; Immunodeficiency diseases

Introduction

Although potent highly active antiretroviral therapy (HAART) has decreased the mortality in HIV-1-infected individuals (Palella, Jr. et al., 1998), the continuous suppression of HIV-1 replication has been associated with reconstitution of immune response to other pathogens but not to HIV-1 (Autran et al., 1997). In fact, prolonged HAART is associated with life-threatening toxicity and decreases virus-specific immune responses (Gray et al., 1999; Ogg et al., 1999; Pitcher et al., 1999). Alternative approaches such as structured therapy interruption (STI) of HAART have been attempted in the clinical management of HIV-1 infection (Lori and Lisziewicz, 2001) with the dual

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purpose of decreasing drug toxicity and increasing virus-specific responses through autovaccination (viral rebound after STI). However, although STI is associated with an increase in virus-specific immune responses, the long-term benefit of STI in patients with long-standing HIV-1 infection is uncertain (Davey, Jr. et al., 1999; Ortiz et al., 1999). The quality and/or quantity of the HIV-1-specific immune response increased by viral rebound may not be sufficient to maintain immune control of viral replication in the absence of HAART. The importance of both virus-specific CD8+ and CD4+ T-cell responses have been demonstrated in primary HIV-1 infection (Koup et al., 1994; Rosenberg et al., 1997) as well as in patients infected with HIV-1 who maintain stable CD4+ T-cells and control viremia in the absence of HAART (Pitcher et al., 1999; Rosenberg et al., 2000). In the SIVmac macaque model of HIV-1 infection of humans, the importance of CD8+ T-cells has been demonstrated indirectly by depletion studies (Jin et al., 1999; Matano et al., 1998; Schmitz et al., 2000). In the same model, the relevance of virus-specific CD4+ T-cells has been inferred by the finding of an inverse correlation between virus plasma level and the size of CD4+ T-helper responses in infected macaques (Hel et al., 2000, 2002a, 2002b; Tryniszewska et al., 2002) as well as by the fact that adoptive transfer of autologous naive CD4+ T-cells ameliorates control of viral replication (Villinger et al., 2002).

Thus, it is logical to infer that increasing the virus-specific CD4+ and CD8+ T-cell response and perhaps even more importantly the virus-specific CD4+ T-cells may be necessary to spare ART treatment of HIV-1-infected individuals. The loss of CD4+ T-lymphocytes in long-standing HIV-1/SIV infection coupled with defects in the number and/or function of virus-specific CD4+ (Musey et al., 1999; Rosenberg et al., 1997) and CD8+ T-cells (Appay et al., 2000; Goepfert et al., 2000; Hel et al., 2001a; Shankar et al., 2000; Vogel et al., 2001) represents, however, serious limitations for immune intervention. Therefore, immune-based strategies need to be able to replenish CD4+ T-helper responses and possibly broaden the immune responses to subdominant epitopes recognized by CD8+ T-cells.

For this purpose, several vaccine modalities need to be assessed and compared for their relative immunogenicity in HIV-1-infected individuals. Among the live vectors, thus far, only a canarypox-based recombinant HIV-1 vaccine (ALVAC-HIV-1) has been tested in a phase I trial in humans and has been demonstrated to be safe and immunogenic. Vaccination with ALVAC-HIV-1 of HAART-treated patients resulted in an increase of virus-specific cellular immune response, which in turn was associated, in a subset of patients, with transient suppression of plasma virus level (Jin et al., 2002; Markowitz et al., 2002).

In the SIVmac model to date, both the canarypox-based SIV (ALVAC-SIV) and the NYVAC-based SIV vaccine candidates have proven to be immunogenic in naive (Benson et al., 1998; Hel et al., 2001a, 2001b; Pal et al., 2001) as well as in infected macaques (Hel et al., 2000, 2002b; Tryniszewska et al., 2002). Both the ALVAC-SIV-*gag-pol-env* (*gpe*) and the NYVAC-SIV-*gpe* vaccine candidates are able to induce equivalent levels of virus-specific CD4+ and CD8+ T-cells in macaques treated with ART during primary infection (Hel et al., 2002b). However, in both those studies the role of the vaccine-induced responses in viremia containment remained elusive since early initiation of ART alone was associated with viremia containment following drug suspension. A similar finding was also reported in other studies in the SIVmac model (Lifson et al., 2000; Lori et al., 2000; Mori et al., 2000; Tsai et al., 1995) as well as in HIV-1-infected individuals treated with ART alone during primary infection (Markowitz et al., 1999; Rosenberg et al., 2000). Encouragingly, however, in macaques with long-standing infection following vaccination either with NYVAC-based SIV vaccines (Tryniszewska et al., 2002) or with the ALVAC-SIV-*gpe*-based vaccine (our unpublished results), viral plasma levels remained significantly lower in the vaccinated than control macaques after cessation of ART, suggesting that vaccination prior to ART suspension may provide clinical and virological benefit. However, the containment of viremia in these macaques was transient (Tryniszewska et al., 2002), suggesting that more immunogenic regimens may be necessary to obtain protracted viremia containment in the absence of ART.

In naive macaques, prior immunization with DNA greatly enhanced the immunogenicity of a recombinant poxvirus-based NYVAC-SIV-*gpe* vaccine (Hel et al., 2001b) and resulted in viremia containment following SIVmac251 mucosal challenge (Hel et al., 2002a). Similarly, when DNA was used to prime and recombinant fowlpox or modified Ankara virus (MVA) to boost, a higher immune response was obtained (Amara et al., 2001; Hanke et al., 1999; Kent et al., 1998; Robinson et al., 1999). Prior vaccination with DNA was demonstrated to broaden cytotoxic T-lymphocyte (CTL) responses (Amara et al., 2001; Hel et al., 2001b) and, importantly, to increase significantly virus-specific CD4+ T-helper cells, which in turn inversely correlated with virus plasma level in macaques vaccinated with the DNA/NYVAC immunization regimen (Hel et al., 2002a).

With the aim of improving the immunogenicity of vaccines in SIV-infected macaques, we designed immunization regimens to directly compare the relative immunogenicity of a DNA/poxvirus to a poxvirus/poxvirus immunization in Mamu-A*01-positive, SIVmac251-infected rhesus macaques. These macaques were able to contain naturally viremia but were nevertheless treated with ART to further minimize and to avoid fluctuation in viral replication that might affect the immune response to vaccination. Immunization with a plasmid expressing the *gag/pol* genes of SIVmac239 (pcDNA-SIV-*gp*) and followed with a recombinant fowlpox (FP) construct expressing the SIVmac251 *gag/pol* genes (FP-SIV-*gp*) (Jenkins et al., 1991) was compared to a regimen of two FP-SIV-*gp* immunizations.

Our purpose was to determine the relative ability of these immunization regimens to induce/expand Mamu-A*01-re-

Table 1
CD4+ T-cell counts at the time of vaccination

Group	Animal	Duration of SIVmac251 infection (months)	Prior vaccination	CD3+/CD4+ T-cells/mm^3 at first immunization	CD3+/CD4+ T-cells/mm^3 at second immunization
A	636	42	NYVAC-SIV-*gpe*	1107	1093
	649	42	NYVAC	1004	1311
	680	31	NYVAC	1062	1251
	682	31	NYVAC	1122	1145
B	645	42	NYVAC	609	963
	650	42	NYVAC-SIV-*gpe*	1152	2506
	681	31	NYVAC	1365	1755
	706	31	NYVAC	1197	1235

stricted SIV-specific CD8+ and CD4+ T-helper cells and the ability of these cells to produce cytokines and the effector function of virus-specific CD8+ T-cells and to assess in parallel the durability of the virus-specific immune responses generated by the two immunization regimens. Since all these SIVmac251-infected macaques were able to contain naturally viremia even in the absence of ART treatment (long-term nonprogressors), there was no plan to evaluate the relative efficacy of these vaccine regimens on viremia containment following ART cessation.

Results

Vaccination with the DNA-SIV-gp and FP-SIV-gp combination induced higher frequency of functional $Gag_{181\text{-}189}$ CM9-specific CD8+ T-cells than the FP-SIV-gp vaccination alone

The eight SIVmac251-infected rhesus macaques enrolled for the study contained viremia in the absence of ART (long-term slow progressors) and had normal CD4+ T-cell counts. The previous treatment of these macaques is summarized in Table 1. Even though these macaques had undetectable viremia, they were treated with ART to minimize possible fluctuation in viral replication that may not have been measurable by the plasma RNA copy assay and to assess more carefully the effect of vaccination in the context of ART. Viremia remained below the detection limit during the entire immunization period (data not shown). Macaques were divided into two groups of four macaques each and immunized with plasmid DNA (DNA-SIV-*gp*) encoding the Gag and Pol SIV proteins and with 2×10^8 pfu of recombinant FP-SIV-*gp* encoding the same proteins 8 weeks later (group A), whereas the remaining macaques (group B) received two immunizations with FP-SIV-*gp* at the same dose at the time indicated in Fig. 1.

Because all the macaques included in this study carried the MHC I Mamu-A *01 molecule, Gag-specific CD8+ T-cells were quantitated at first in blood using the $Gag_{181–189}$ CM9 Mamu-A *01 tetrameric complexes. Whereas a negligible increase in this CD8+ T-cell population was found following inoculation with DNA-SIV-*gp*, in the animals of group A, a significant increase in the frequency of tetramer-staining CD8+ T-cells was observed in all macaques from group B within a few weeks from immunization with the FP-SIV-*gp*. This response reached peak values at week 2, up to 17.78 and 15.45% in animals 645 and 650, respectively (Fig. 2A), and in most macaques remained higher than the prevaccination level until the second vaccination. The second immunization with FP-SIV-*gp* in the same group of macaques (group B) did not result in greater expansion of this Gag-specific CD8+ T-cell response (Fig. 2A). Surprisingly, however, a much greater increase in the Gag-specific tetramer-staining population of CD8+ T-cells was observed in three of the four macaques of group A, which had been previously immunized with DNA, reaching frequencies in blood of 24.4, 45.1, and 38.2% of the total CD8+ T-cells in macaques 636, 649, and 680, respectively. These frequencies remained higher than the level preceding immunization in these three animals for as long as 5 months following the last immunization (Fig. 2A).

Statistical analysis of the frequency of these responses in macaques from both groups using the repeated measures

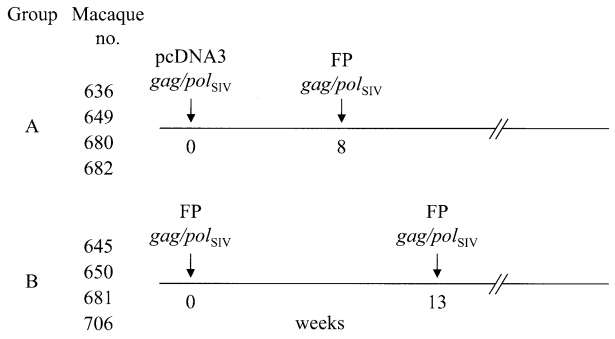
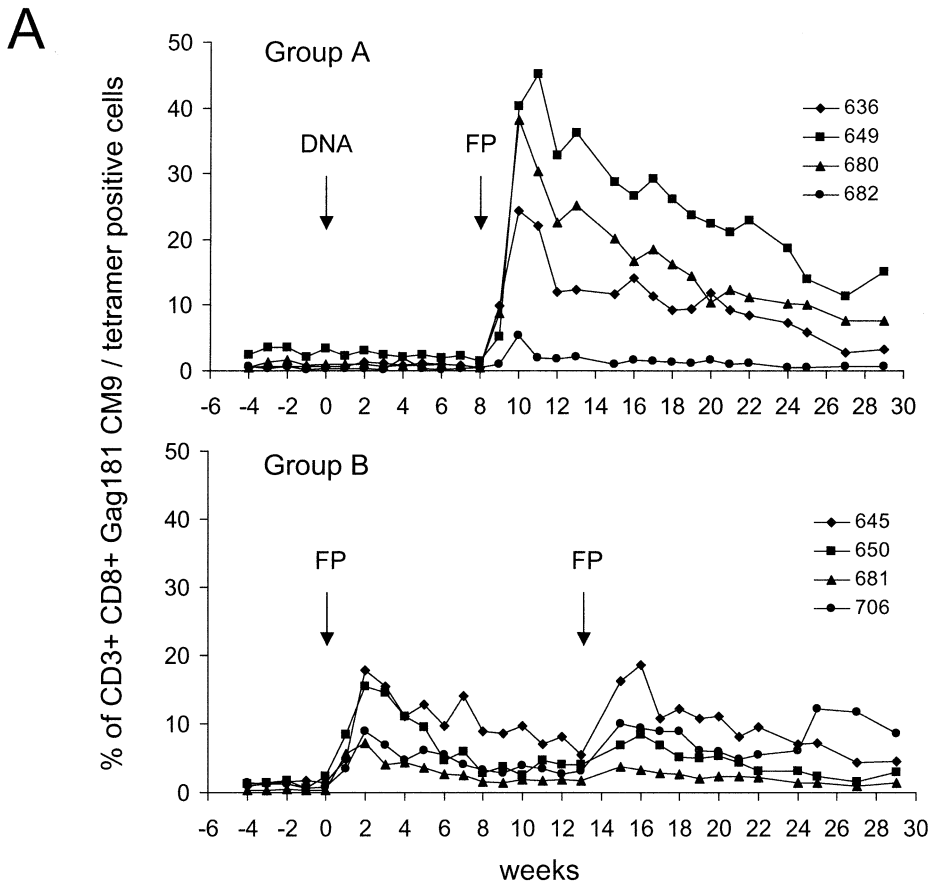
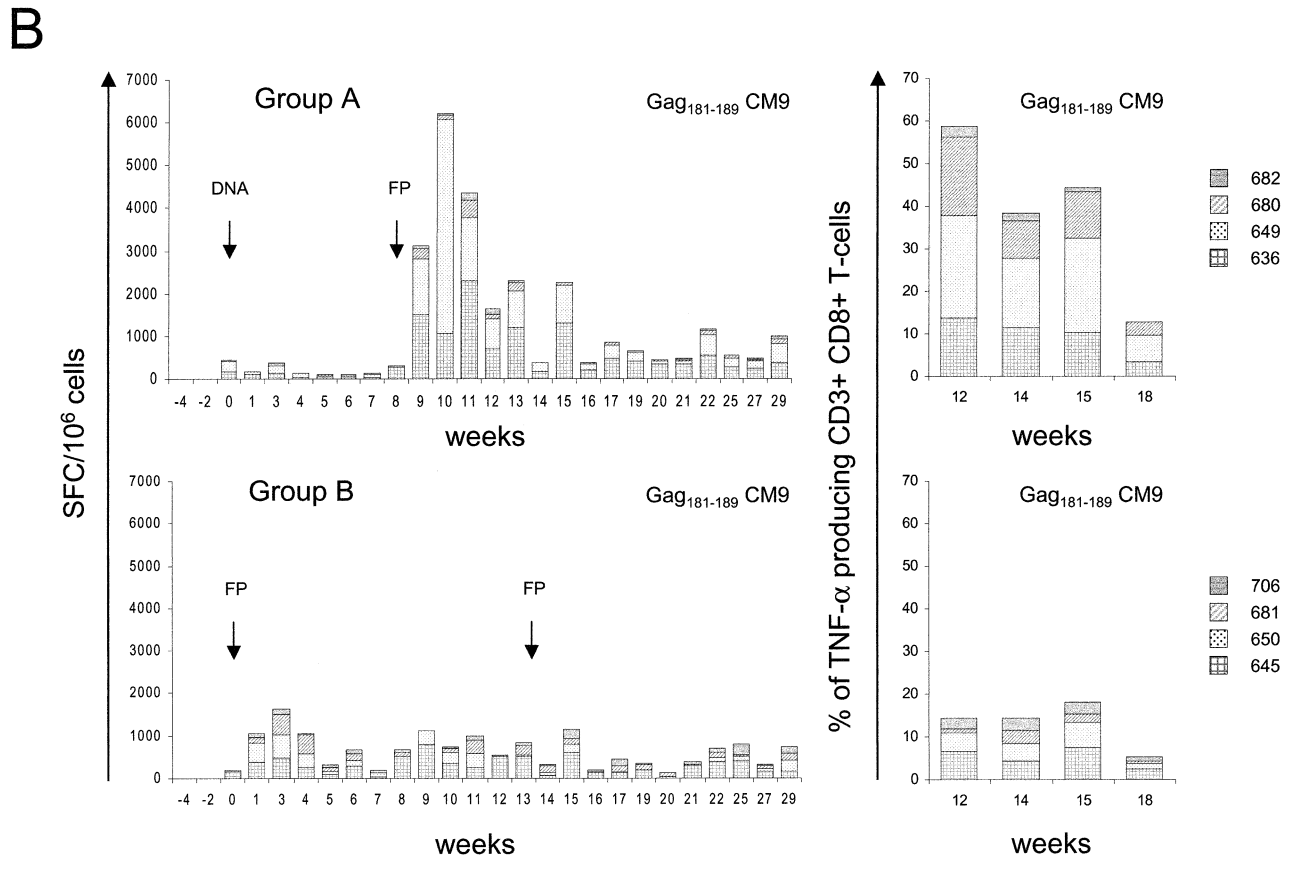


Fig. 1. Study design. The macaques in each group were immunized twice at the times indicated. ART was initiated 4 weeks before vaccination and maintained during the entire experiment. pcDNASIV*gp* and FPSIV*gp* recombinants contained the complete *gag/pol* genes from SIVmac239. All macaques were Mamu-A*01-positive and their previous treatment is summarized in Table 1.


A
Group A
DNA
FP
636
649
680
682
Group B
FP
FP
645
650
681
706
% of CD3+ CD8+ Gag181 CM9 / tetramer positive cells
weeks


B
Group A
Gag181-189 CM9
DNA
FP
Group B
FP
FP
SFC/10^6 cells
% of TNF-α producing CD3+ CD8+ T-cells
682
680
649
636
706
681
650
645
weeks

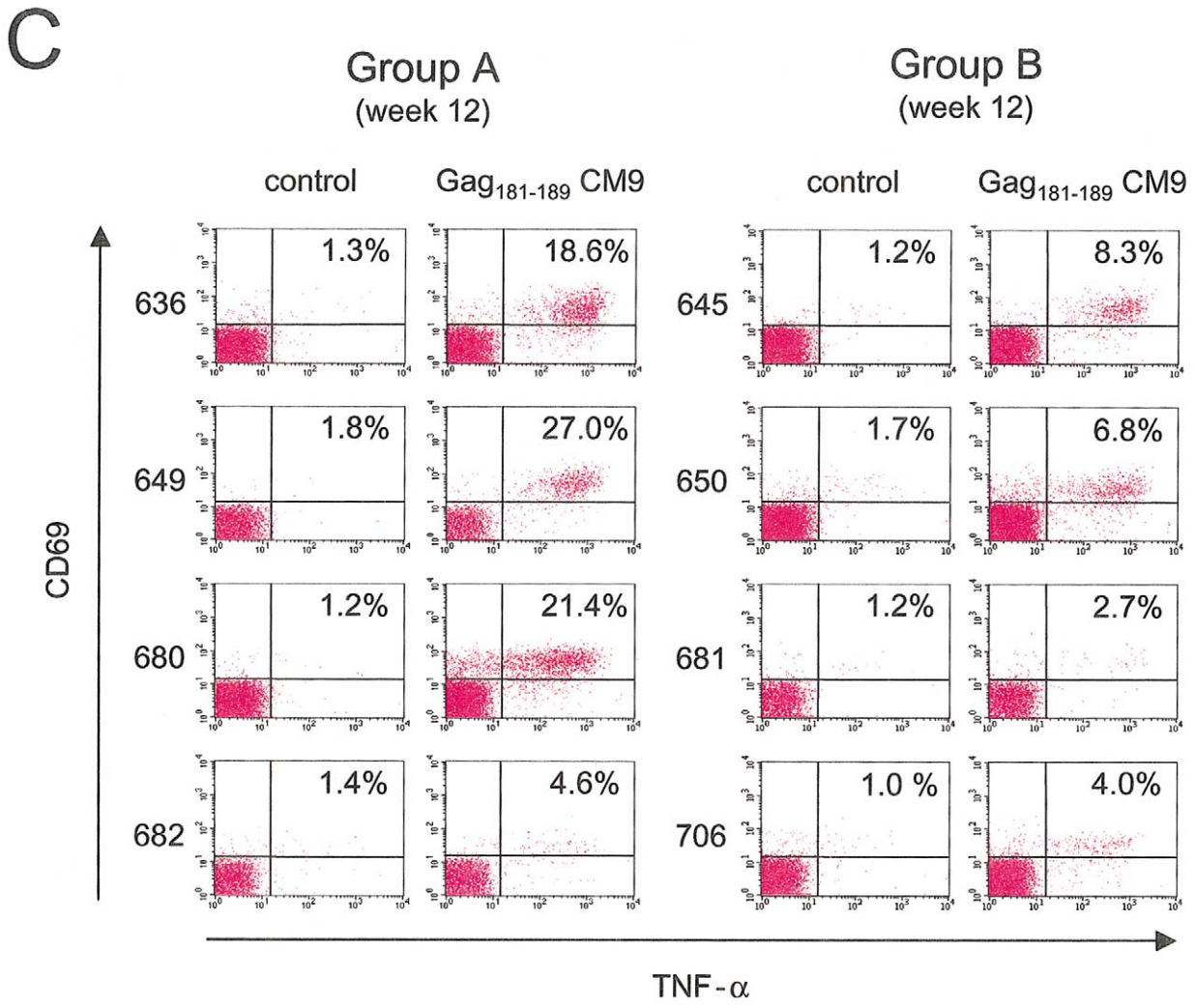
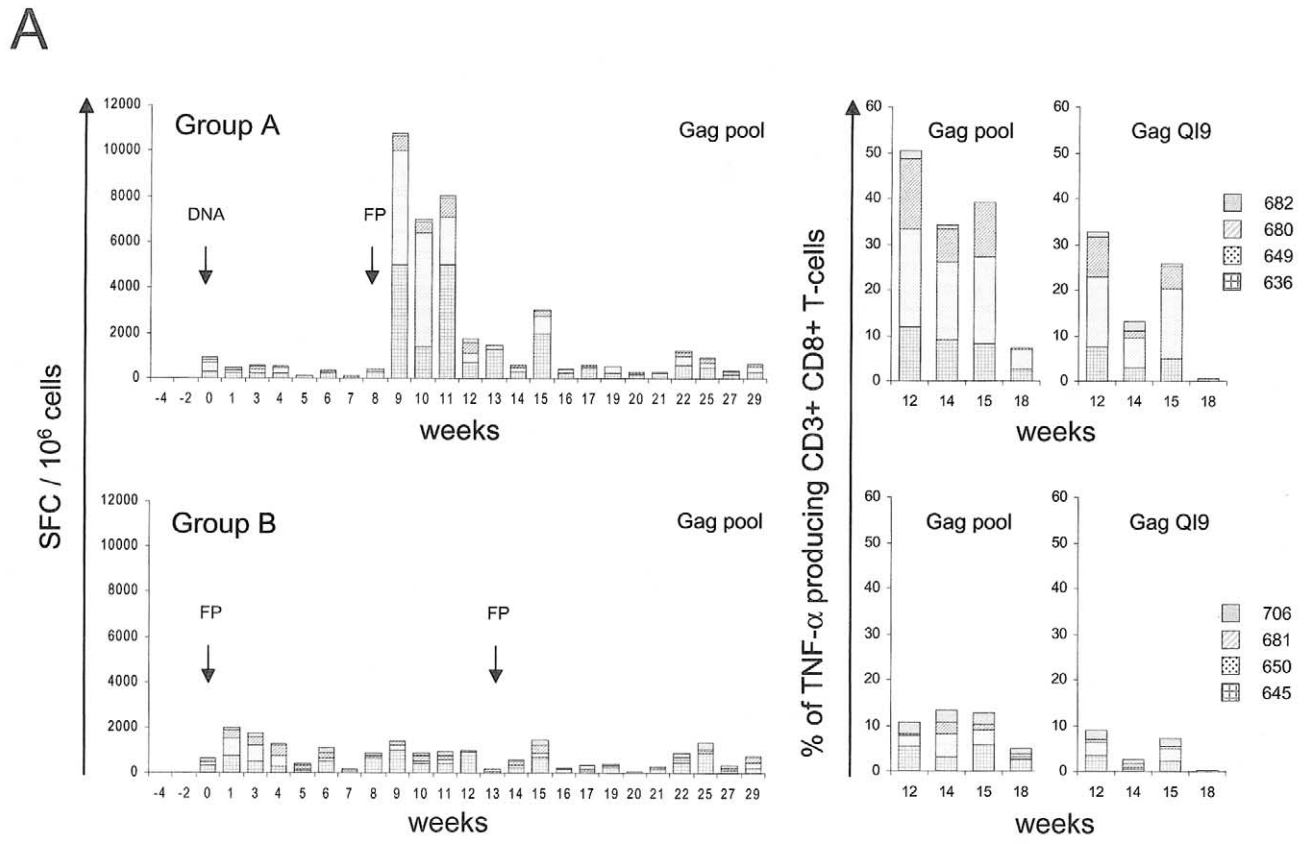


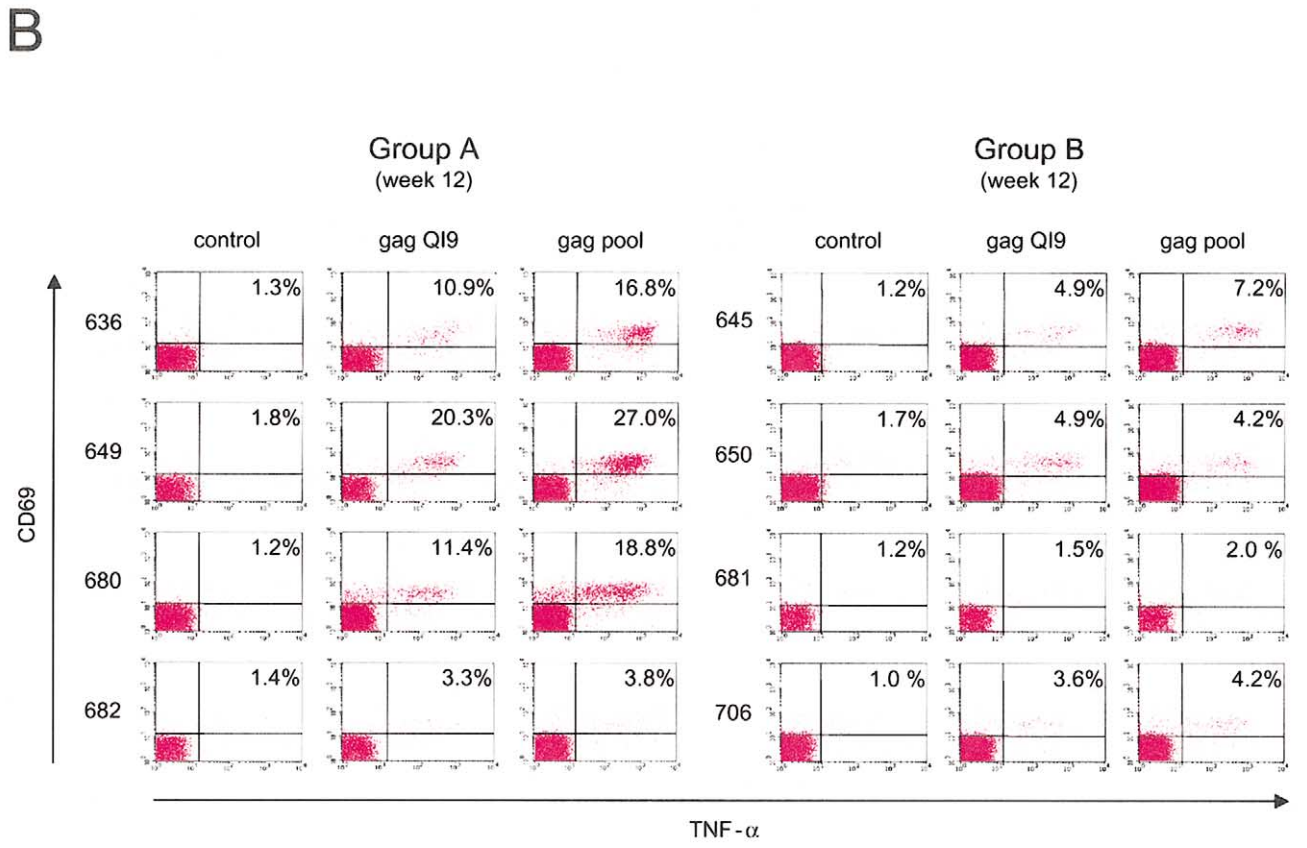
Fig. 2 (*continued*)

analysis of variance of the arc-sine tetramer-positive percentage indicated that prior DNA immunization contributed significantly to the expansion of this response (compare the first FP-SIV-*gp* administration in groups A and B, $P = 0.018$). The increase in this population of Gag-specific cells induced by the combination of DNA/FP-SIV-*gp* was also significantly greater than that obtained after the second FP-SIV-*gp* immunization of group B macaques ($P = 0.0074$). Although both immunization regimens resulted in the maintenance of a high frequency of Gag-specific CD8+ T-cells for several weeks postimmunization, the blood levels of these cells remained higher over time in macaques from group A than from group B, suggesting that the DNA/FP-SIV-*gp* combination may elicit/expand a higher number of memory T-cells. Prior immunization of two of these macaques, 636 in group A and 650 in group B, did not apparently influence these results since the former had intermediate response and the latter did not differ from the remaining macaques in group B.

Because binding of CD8+ T-cells to the specific tetramer does not necessarily correlate with their ability to produce cytokines, we tested the ability of these cells to produce IFN-γ by ELISPOT and TNF-α by intracellular cytokine staining. After in vitro stimulation with the Mamu-A*01-restricted SIVmac251-specific $Gag_{181–189}$ CM9 peptide, IFN-γ-secreting spot-forming cells (SFCs) were counted and the average number per million assessed after subtracting the number of cells producing IFN-γ counted in

Fig. 2. Functional virus-specific T-cell response to $Gag_{181–189}$ CM9 epitope induced by vaccination. Results of animals of groups A and B are shown. (A) The frequency of CD3+ CD8+ T-cells was determined by tetramer staining using the $Gag_{181–189}$ CM9 peptide. (B, left) IFN-γ production was measured after specific antigenic stimulation with $Gag_{181–189}$ CM9 peptide in an ELISPOT assay; (right) TNF-α production was determined by intracellular cytokine staining assay following stimulation with $Gag_{181–189}$ CM9 peptide. (C) TNF-α production raw data at week 12. The percentage of CD69 TNF-α-secreting cells is shown at the top right in each panel. Unstimulated control cells are shown at the left side of each group.


A
Group A
Gag pool
DNA
FP
SFC / 10^6 cells
weeks
Group B
Gag pool
FP
FP
weeks
% of TNF-α producing CD3+ CD8+ T-cells
Gag pool
Gag QI9
682
680
649
636
weeks
Gag pool
Gag QI9
706
681
650
645
weeks


B
Group A
(week 12)
Group B
(week 12)
control
gag QI9
gag pool
636
1.3%
10.9%
16.8%
649
1.8%
20.3%
27.0%
680
1.2%
11.4%
18.8%
682
1.4%
3.3%
3.8%
645
1.2%
4.9%
7.2%
650
1.7%
4.9%
4.2%
681
1.2%
1.5%
2.0 %
706
1.0 %
3.6%
4.2%
CD69
TNF-α

the absence of stimulation (average value of medium alone in our conditions is 25 SFC/10^6 cells). DNA immunization did not increase significantly the number of IFN-γ-producing cells above the preimmunization level in any of the macaques in group A (Fig. 2B, left panels). The first immunization of group B macaques with FP-SIV-*gp* increased the frequency of $Gag_{181–189}$ CM9-specific SFCs in three of the four macaques and this response was not boosted by the second FP-SIV-*gp* immunization (Fig. 2B). However, a remarkable boost in the number of IFN-γ-producing cells occurred in group A within a week postimmunization with the FP-SIV-*gp* in three of the four macaques. Thus, the IFN-γ responses to the $Gag_{181–189}$ CM9 peptide mirrored closely the results obtained with the $Gag_{181–189}$ CM9 Mamu-A*01 tetrameric complex.

To further confirm these findings with an additional independent assay, intracellular TNF-α staining was performed following in vitro stimulation with the $Gag_{181–189}$ CM9-specific peptide over several weeks after the second immunization. A high number of CD8+ T-cells producing TNF-α following peptide-specific stimulation was found in the same three of four macaques from group A that had a high level of blood tetramer-positive cells (Fig. 2B, upper right panel). In macaques from group B, the response to the second vaccination was lower (Fig. 2B, right lower panel, and Fig. 2C). Thus, independent assays demonstrated that the vaccine-induced expansion of the Gag-specific response was higher and longer lasting in the macaques previously immunized with DNA (group A) than in macaques immunized at first with FP-SIV-*gp* (group B).

Functional response to subdominant epitopes induced by vaccination

To assess whether the DNA/FP-SIV-*gp* vaccine modality combination could also potentiate the response to subdominant epitopes, an overlapping peptide pool (15 amino acids overlapping 11 amino acids) encompassing the entire Gag protein of SIV and the subdominant peptide, QI9, was used in ELISPOT and intracellular cytokine staining assays. Assays using overlapping Env peptides were also performed as a control since the Env protein was not included in the vaccine. An overall higher number of cells producing IFN-γ was found following stimulation with the *gag* pool than with the $Gag_{181–189}$ CM9 peptide alone (compare Fig. 3A, left panel, to Fig. 2B), suggesting that other Gag epitopes were also recognized by the T-cells of the immunized macaques. Similarly, the frequency of TNF-α-producing cells following in vitro stimulation with the subdominant Gag QI9 peptide was higher in macaques of group A immunized with the combination of DNA and FP-SIV-*gp* than in macaques of group B that received two FP-SIV-*gp* vaccinations (Fig. 3A, right top panel, and Fig. 3B). No significant increase of ELISPOT response to the Env overlapping peptides was observed, as expected (data not shown). Thus, the combination of these vaccine modalities increased not only dominant but also subdominant Gag-specific CD8+ T-cell responses.

*Ex vivo cytolytic activity to dominant and subdominant Mamu-A*01-restricted epitopes in the immunized macaques*

Since all the assays performed above assessed the ability of antigen-specific cells to produce cytokines but did not directly demonstrate their cytolytic function, ^{51}Cr release assays were performed in parallel to the other immunological assays during the immunization regimens (Fig. 4). In animals of group A, ex vivo cytolytic T-cells specific for the $Gag_{181–189}$ CM9 or Gag QI9 epitope were not detected at 5 weeks following immunization with DNA. However, by 5 weeks after the second immunization with FP-SIV-*gp* (week 13), ex vivo cytolytic activity with both specificities was readily detected in the blood of most macaques (three of four) in this group and a low level of this cytolytic activity to the dominant $Gag_{181–189}$ CM9 peptide persisted over time (Fig. 4). In contrast, in macaques from group B only, ex vivo cytolytic activity to the dominant $Gag_{181–189}$ CM9 epitope was detected at 5 weeks from the first immunization and 2 weeks after the second immunization (week 15).

All together, these results demonstrate that, although ex vivo CTL activity could be induced by FP-SIV-*gp* immunization alone, macaques that received DNA before the FP-SIV-*gp* vaccine had a higher level of and more durable ex vivo CTLs to the dominant $Gag_{181–189}$ CM9 and also exhibited ex vivo cytolytic activity to the subdominant Gag QI9 epitope.

Virus-specific CD4+ T-cell responses induced by vaccination

CD4+ T-cells are depleted in HIV-1/SIV infection and they are important in the maintenance of CD8+ T-cell effector activity (Zajac et al., 1998). In the case of SIVmac251 infection of macaques, virus plasma level correlates inversely with virus-specific CD4+ T-cell response, sug-

Fig. 3. Virus-specific T-cell response to Gag pool peptides and to the subdominant Gag Q19 epitope induced by vaccination. Results of animals of groups A and B are shown. (A, left) IFN-γ production was measured after specific antigenic stimulation with a pool of Gag peptides in an ELISPOT assay; (right) TNF-α production was determined by intracellular cytokine staining assay following stimulation either with a pool of Gag peptides (left side) or with the Gag Q19 peptide (right side). (B) TNF-α production raw data for week 12. The percentage of CD69 TNF-α-secreting cells is shown at the top right in each panel. Unstimulated control cells are shown at the left side of each group.

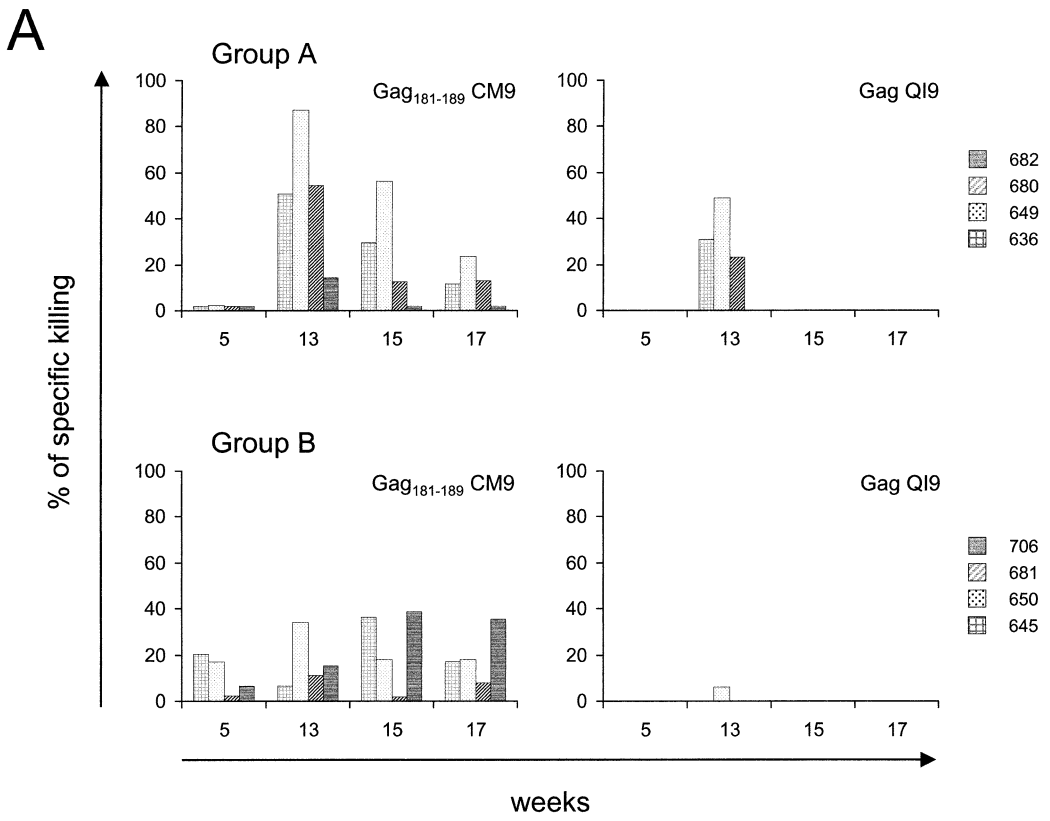


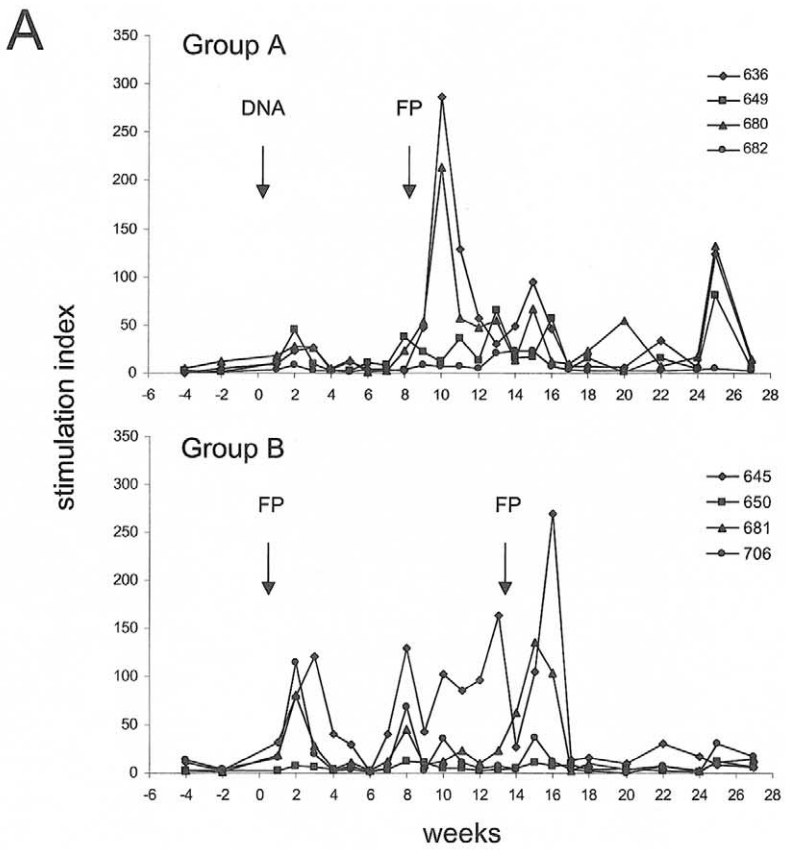
Fig. 4. Virus-specific CTLs. Cytotoxic activity at a 50:1 effector:target ratio was determined at 5, 13, 15, and 17 weeks after the first immunization. PBMCs were stimulated with IL-2 and immortalized target B-cells were pulsed either with $Gag_{181–189}$ CM9 (top) or Gag Q19 peptide (bottom).

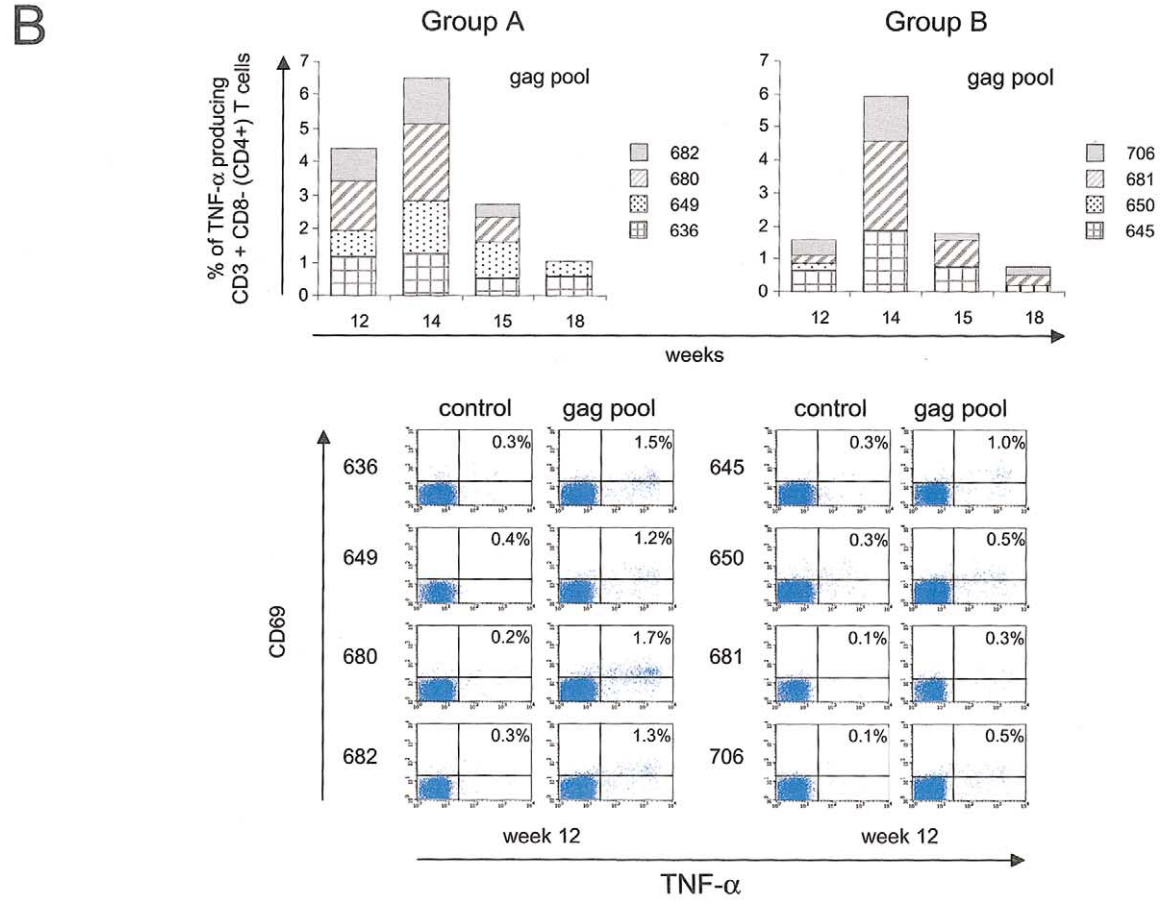
gesting the role of CD4+ T-cells in the immune control of viremia (Hel et al., 2000). Functional analysis of SIV-specific CD4+ T-cells was therefore performed by measuring both lymphocyte proliferation responses (LPRs) to SIV antigens and intracellular cytokine staining following in vitro stimulation with a pool of SIV Gag overlapping peptides (15-mers with 11-amino-acid overlap) in macaques from groups A and B.

Both groups of macaques exhibited proliferative response to p27 Gag following FP-SIV-*gp* vaccination, and these responses were very high [stimulation index (SI) >200] in some macaques from group A. However, the heterogeneity in this response among macaques did not allow for a proper statistical evaluation of a difference between group A and group B macaques (Fig. 5A). As expected, LPRs to gp120 were low or negative at the start of immunization and were unaffected by vaccination (data not shown).

An analysis of the frequency of CD4+ T-helper cells producing TNF-α following Gag pool peptide stimulation was performed in blood cells from macaques in both groups. In all macaques from group A, discrete populations of CD4+ T-cells were still clearly detectable at 4 weeks after immunization with FP-SIV-*gp* (Fig. 5, top panel). In macaques from group B, Gag-specific CD4+ T-cell responses were also measured as late as 12 weeks after the first immunization with FP-SIV-*gp* (week 12). Following the second FP-SIV-*gp* immunization at week 13, the responses were increased but they appeared less durable than those induced by DNA-SIV-*gp*/FP-SIV-*gp* vaccination (Fig. 5, lower panels) in macaques in group A. In fact, by 2 weeks postimmunization (week 15) the extent of the responses was equivalent to the preimmunization level in macaques from group B. Thus, Gag-specific CD4+ T-cell responses appeared to be more durable in macaques immunized with DNA prior to FP-SIV-*gp* vaccination.

Fig. 5. CD4+ T-cell response to Gag. (A) CD4+ T-cell response was determined by lymphoproliferation assay and shown for individual animals. Standard deviation is also shown. (B) TNF-α production was determined by intracellular cytokine staining assay of CD3+ CD8− (CD4+) T-cells after in vitro stimulation with a pool of Gag peptides and determined for weeks 12, 14, 15, and 18 (top). Bottom panels show TNF-α production raw data for week 12. The percentage of CD69 TNF-α-secreting cells is shown at the top right in each panel.


A
Group A
DNA
FP
636
649
680
682
stimulation index
Group B
FP
FP
645
650
681
706
weeks


B
Group A
Group B
gag pool
gag pool
% of TNF-α producing CD3 + CD8- (CD4+) T cells
682
680
649
636
706
681
650
645
12
14
15
18
weeks
control
gag pool
control
gag pool
CD69
636
649
680
682
645
650
681
706
0.3%
1.5%
0.4%
1.2%
0.2%
1.7%
0.3%
1.3%
0.3%
1.0%
0.3%
0.5%
0.1%
0.3%
0.1%
0.5%
week 12
week 12
TNF-α

Discussion

A therapeutic vaccination approach able to ameliorate immune control of HIV-1 infection in the absence of ART will, minimally, require the restoration of an adequate level of virus-specific CD4+ T-cell helper function in addition to an expansion of virus-specific CD8+ T-cells. The expanded/induced virus-specific CD8+ T-cell response should also be of sufficient breadth to prevent immune escape during viral rebound. At present, limited data in both humans and macaques also support the notion that antibodies able to neutralize the endogenous virus may be of great importance (Montefiori et al., 2001; Tryniszewska et al., 2002). The only live recombinant poxvirus vector-based HIV-1 vaccine tested to date in therapeutic intervention in humans is based on the canarypox vector. At the dose tested, the immune response induced by ALVAC-HIV-1, albeit sizeable, was not sufficient to confer sustained immune control of HIV-1 replication (Jin et al., 2002; Markowitz et al., 2002). Both ALVAC-SIV-based and the highly attenuated NYVAC-SIV-based vaccine candidates have been tested in naive (Benson et al., 1998; Hel et al., 2002a; Pal et al., 2001; Santra et al., 2002) and infected (Hel et al., 2000; Tryniszewska et al., 2002) (our unpublished results) macaques treated with ART during primary or long-standing SIVmac251 infection. Whereas immune control on viral replication could not be assessed in primary infection because of the effect of ART per se (Hel et al., 2000), in macaques with long-standing SIVmac251 infection, both vaccines exhibited an apparent equivalent immunogenicity and lowered the set-point viremia after ART suspension (Tryniszewska et al., 2002) (our unpublished results). However, the immune control of viral replication was not durable and within 1 year plasma viral level progressively also increased in the vaccinated macaques.

One limitation of a poxvirus-based single vaccine modality is that the induction of immunity to the vector can interfere with the ability to induce/expand memory response to the desired antigen after repeated immunizations, as demonstrated in the case of vaccinia virus (Harrington et al., 2002). Therefore, the use of mixed modalities needs to be explored as a means to improve both the qualitative and quantitative immune responses induced by each independent vaccine.

Naked DNA immunization induces long-term memory response (Gurunathan et al., 2000); however, its immunogenicity is lower in primates than in mice. Early studies in a murine model of malaria suggested that DNA poxvirus-based vaccine mixed modalities were more immunogenic and afforded better protection (Ramshaw and Ramsay, 2000; Schneider et al., 1998; Sedegah et al., 1998). In naive macaques, the DNA prime/live-vector boost strategy has elicited better immune responses than DNA or poxvirus alone (Amara et al., 2001; Hanke et al., 1999; Hel et al., 2001b; Kent et al., 1998; Lu et al., 1996; Robinson et al., 1999). Importantly, the mixed DNA/poxvirus vaccine modality was shown to elicit memory CD4+ T-helper responses to a much higher extent than either the poxvirus-based vaccine or DNA alone (Hel et al., 2001b). However, whether prior DNA immunization in the context of a therapeutic vaccination could also potentiate a poxvirus-based vaccine was previously unknown and not necessarily predictable.

The data presented here demonstrate that in long-term nonprogressor SIVmac251-infected macaques the DNA/FP vaccine modality combination is also able to induce significantly higher, broader, and more durable virus-specific CD4+ T-cell and CD8+ T-cell responses than either FP or DNA alone.

A possible explanation of this phenomenon is that DNA immunization increases both CD4+ and CD8+ T-cell responses below the threshold of detection and only after FP-SIV-*gp* vaccination is this subliminal expansion of antigen-specific cells revealed. In the case of the second immunization in animals of group B, the increase of SIV-specific immune responses would be lower than in group A because the immune response would be partially directed to FP proteins, as demonstrated elegantly in the murine model using a lymphocytic choriomeningitis virus dominant epitope in the context of a vaccinia recombinant virus (Harrington et al., 2002). However, the extent of this phenomenon may be reduced in the case of vectors that do not replicate, as in the case of the fowlpox vector in murine cells. In the case of DNA alone, the low immunogenicity is probably related to the relatively inefficient expression of DNA in tissues. Nevertheless, in the context of SIVmac infection controlled by ART, the DNA immunization may be exploited to circumvent the vector-directed immunity in regimens that alternate the use of these vaccine modalities.

Orthopoxvirus and avipoxviruses all have immunostimulatory capacity (Fachinger et al., 2000). In particular, the canarypox-derivative ALVAC vector has been recently demonstrated to infect dendritic cells to induce their maturation (Ignatius et al., 2000) and elicit strong HIV-1-specific CD8+ and CD4+ responses in cell cultures of HIV-1-infected peripheral blood mononuclear cells (PBMCs) (Engelmayer et al., 2001). Importantly, ALVAC-infected dendritic cells can directly stimulate and expand virus-specific CD4+ T-cells in vitro and the expansion of CD8+ T-cells is dependent on the presence of CD4+ T-helper cells (Engelmayer et al., 2001). Fowlpox is also able to infect dendritic cells and stimulate antigen-specific T-cells in vitro (Brown et al., 2000), and here we demonstrate that this vector is able to expand both virus-specific CD4+ and CD8+ T-cells in vivo. Because CD4+ T-cells are the target of HIV-1 and because of their importance in the generation and maintenance of broad virus-specific CD8+ T-cells (Kalams et al., 1999; Matloubian et al., 1994; Zajac et al., 1998), poxvirus-based HIV-1 vaccines may be ideal modalities in combination with DNA to replenish the lost CD4+ T-helper cells in HIV-1-infected individuals. A similar approach has been used in other viral infections of humans (Chen et al., 2000) and may also be effective in cancer immunotherapy.

Materials and methods

Cells

Specific pathogen-free primary chick embryo fibroblasts were maintained in Dulbecco's minimum essential medium supplemented with 10% heat-inactivated calf serum (Gibco, Life Technologies, Grand Island, NY), 100 U/ml penicillin, and 100 μg/ml streptomycin. Monkey PBMCs were grown in RPMI-1640 medium with L-glutamine (Gibco, Life Technologies) containing 10% heat-inactivated human serum (Sigma, St. Louis, MD). Monkey B-cells were transformed by Herpes Papio virus S594 and immortalized cells were expanded and maintained in RPMI supplemented with 15% heat-inactivated fetal calf serum (Gibco, Life Technologies) and penicillin and streptomycin.

Plasmid and recombinant fowlpox virus

Plasmid pcDNA3*gag/pol*$_{SIV}$ (DNA-SIV-*gp*), containing the *gag/pol* genes of SIVmac239 and the constitutive transport element of the Mason–Pfizer monkey virus, was prepared as already described (Radaelli et al., 2002) and amplified using the endofree DNA extraction kit (Qiagen, Valencia, CA). Viral stocks were prepared on primary chick embryo fibroblasts, purified on a sucrose gradient, titered, and used for animal immunization. The correct expression of both the *gag/pol* genes of pcDNA3-SIV*gag/pol* (Radaelli et al., 2002) as well as of the FP-SIV-*gp* (FP74) recombinant were previously demonstrated (Jenkins et al., 1991).

Animals

Eight colony-bred rhesus macaques (*Macaca mulatta*), obtained from Covance Research Products (Alice, TX), were infected with the same strain of SIVmac251 (561) (Pal et al., 2001) and treated daily with a combination of potent antiviral drugs: 20 mg/kg/day sc of (*R*)-9-2-phosphonylmethoypropyladenine (PMPA) (started at day 15) and 5 mg/kg/day ip of didanosine (DDI; Videx, Bristol-Meyers Squibb, Princeton, NJ). The treatment was continued for 6 months and the macaques were vaccinated with NYVAC-SIV-*gpe* or NYVAC alone during ART (Hel et al., 2000) (for details, see Table 1). Once therapy was suspended, viral rebound occurred in most animals but was subsequently contained as a result of early ART intervention (Franchini, 2002). ART was initiated again for the purpose of this study 1 month before the first immunization and maintained during the entire immunization regimen to minimize fluctuation in viral replication that may have influenced the response to vaccination. All macaques were Mamu-A*01-positive as demonstrated by reverse transcription–polymerase chain reaction (Kuroda et al., 1998) and were housed and handled in accordance with the standards of the American Association for the Accreditation of Laboratory Animal Care.

Immunization protocols

Eight rhesus monkeys were divided into two groups of four animals each. Animals of group A (636, 649, 680, and 682) were immunized with 4 mg/animal of DNA-SIV-*gp* (3 mg im and 1 mg id) and boosted after 8 weeks with a single inoculation of 2×10^8 pfu/animal intramuscularly of FP-SIV-*gp*. Animals in group B (645, 650, 681, and 706) were immunized at first intramuscularly with 2×10^8 PFU/animal FP-SIV-*gp* and boosted after 13 weeks with the same dose of the same vaccine. Blood was collected weekly and PBMCs were isolated by Ficoll. Serum fraction aliquots were also collected and frozen at -80°C until used.

NASBA and CD4+ and CD8+ T-cell counts

RNA detection was performed by the nucleic acid sequence-based amplification (NASBA) technology. Briefly, RNA was extracted from plasma and isothermally amplified using SIVmac251-specific primers. Quantification was made by electrochemiluminescence-chemistry-based probe hybridization system using a coextracted internal standard. The copy number is expressed per hundred microliters of plasma, the detection limit of the assay being 2×10^3 RNA copies.

CD4+ and CD8+ counts were periodically determined on 100 μl of whole blood and by FACS analysis, according to the FACS/Lyse kit (BD Immunocytometry Systems, San Jose, CA) with minor modifications. Briefly, after incubating 10 μl of a mixture containing PerCP-CD4, APC-CD8, PE-CD45, and FITC-CD3 Ab (BD Immunocytometry Systems) for 30 min at room temperature, red cells were lysed by adding 2 ml of FACS/Lyse solution for 15 min. Samples were then centrifuged for 5 min at 1200 rpm at room temperature, washed [1% fetal calf serum (FCS) and 0.05% NaAzide in phosphate-buffered saline (PBS)], resuspended in 500 μl wash buffer, and stored at 4°C until acquired by a FACSCalibur flow cytometer (Becton–Dickinson). The counts were expressed as a percentage of CD3/CD4+ or CD3/CD8+ lymphocytes/μl.

Tetramer staining

The Mamu-A*01 tetrameric complex, refolded in the presence of the Gag$_{181–189}$ CM9 peptide, was kindly supplied by J.D. Altman (Emory University Vaccine Center at Yerkes, Atlanta, Georgia) and conjugated to streptavidin (Molecular Probes, Eugene, OR). Fresh PBMCs (5×10^3) per animal were simultaneously stained with differently conjugated antihuman Abs: PerCP-CD8 (BD Immunocytometry Systems), FITC-CD3ε (BD PharMingen, San Diego, CA), and the PE-conjugated tetrameric complex. PE-CD4 (BD PharMingen) Ab was used for the positive control. After 30 min of incubation in the dark at room temperature, cells were washed with 2% FCS in PBS and fixed with 1% paraformaldehyde in PBS (pH 7.4). Samples

were analyzed on a FACSCalibur flow cytometer (Becton–Dickinson) and cells were sorted and expressed as a percentage of tetramer-positive cells over the total CD8+ T-lymphocyte population.

ELISPOT assay

ELISPOT assay was performed with the monkey-specific IFN-γ U-Cytech kit (Utrecht, The Netherlands), following the manufacturer's specifications. Briefly, after coating with anti-IFN-γ mAb, plates were incubated overnight at 4°C and washed and aspecific sites covered with 2% bovine serum albumin in PBS at 4°C for 3 to 5 h. PBMCs (10^5) were plated per each well in RPMI-1640 containing 5% heat-inactivated human serum and stimulated in triplicate with $Gag_{181–189}$ CM9, Gag Q19, or a pool of Gag or Env peptides, each at 1 μg/ml. Concanavalin A (5 μg/ml) was used as a positive control. After overnight incubation at 37°C in a 5% CO_2 atmosphere, plates were developed and SFCs counted onto a Leitz Diavert inverted microscope.

Intracellular cytokine staining

Intracellular cytokine staining was performed using the anti-TNF-α antibody. Fresh PBMCs (10^6) in 1 ml complete medium were incubated for 1 h at 37°C in the absence or presence of $Gag_{181–189}$ CM9 (10 μg/ml), Gag Q19 (10 μg/ml), or a pool of Gag peptides (2 μg/ml) and in the presence of CD28 and CD49d (1 μg/ml each). After addition of 10 μg/ml Brefeldin A (Sigma), cells were incubated for 5 h at 37°C and processed for surface and intracellular cytokine staining. Briefly, after treatment with 2 mM EDTA and incubation at room temperature for 15 min, cells were washed twice with 1% FCS in PBS, surface-stained with FITC-CD3ε and PerCP-CD8 (10 μl each), washed again, and permeabilized with FACSPerm (BD PharMingen) for 10 min at room temperature in the dark. Following two further washes, cells were intracellular-stained with PE-CD69 (0.006 μg) and allophycocyanin-conjugated-TNF-α (0.4 μg, BD PharMingen), incubated for 25 min at 37°C, fixed with 500 μl 1% paraformaldehyde (Sigma) in PBS, and analyzed by four-color flow cytometry (Becton–Dickinson).

CTL assay

Monkey PBMCs were stimulated overnight with 100 U IL-2 (Roche, Indianapolis, IN) in 1 ml complete medium, washed in HBSS, and plated in triplicate on a 96-well round-bottomed plate. Mamu-A*01-positive Herpes Papio-transformed target cells were stimulated overnight with 10 μg $Gag_{181–189}$ CM9 or Gag Q19 peptides in 1 ml complete medium, labeled for 2 h with 100 μCi ^{51}Cr, and washed in HBSS, and 5×10^3 cells were added to each well. Unstimulated target cells were used as a negative control. Cells were incubated for 6 h at effector-to-target-cell ratios of 50:1, 25:1, 12.5:1, and 6.25:1 and the percentage of released ^{51}Cr was calculated by dividing the difference between the mean counts per minute of experimental and spontaneous release by the difference between the mean counts per minute of total and spontaneous release.

Lymphocyte proliferation assay

Monkey PBMCs, isolated by lymphocyte separation medium (Cappel, ICN Biomedicals Inc., Aurora, OH) from EDTA or heparinized blood, were prepared according to the manufacturer's specifications and plated at 3×10^5/well into flat-bottomed 96-well plates. Cells were stimulated with 1 μg/well of p27 (Advanced BioScience Laboratories, Rockville, MD), 1 μg/well of gp120 (Advanced BioScience Laboratories), or 5 μg/well of concanavalin A (Sigma) as a positive control. On day 3, 1 μC [*methyl*-^{3}H]thymidine (NEN, Life Science Products, Boston, MA) was added to each microculture. Following an 18-h incubation, cells were harvested with a Tomtec cell harvester (Model No. 96, CraMar Technologies, Westminster, CO) and the incorporated radioactivity was determined in a Wallac 1205 Betaplate liquid scintillation counter (Amersham Pharmacia Biotech, Piscataway, NJ). The SI was calculated by dividing the mean counts per minute of [*methyl*-^{3}H]thymidine incorporated into antigen-pulsed cells by the mean counts incorporated into unstimulated cells. Experiments were performed in triplicate for each animal.

Statistical analyses

The percentage of tetramer-positive CD8+ lymphocytes was analyzed by repeated measures analysis of variance applied to the arc-sine-transformed data. All *P* values were two-tailed. The Number Cruncher Statistical System (NCSS, Kaysville, UT), Sigmastat (version 2.0; SPSS, Chicago, IL), and the SAS System for Windows (release 8.00; SAS Institute Inc., Cary, NC) statistical software packages were used for the analyses.

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