MAJOR ARTICLE

Modulation of Vaccine-Induced Immune Responses to Hepatitis C Virus in Rhesus Macaques by Altering Priming before Adenovirus Boosting

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Background. Preventive and therapeutic vaccine strategies aimed at controlling hepatitis C virus (HCV) infection should mimic the immune responses observed in patients who control or clear HCV, specifically T helper (Th) type 1 and CD8^{+} cell responses to multiple antigens, including nonstructural protein (NS) 3. Given the experience with human immunodeficiency virus, the best candidates for this are based on DNA prime, pox, or adenovirus boost regimens.

Methods. In rhesus macaques, we compared NS3-expressing DNA prime and adenovirus boost strategy with 2 alternative priming approaches aimed at modifying Th1 and CD8^{+} responses: DNA adjuvanted with interleukin (IL)–2– and –12–encoding plasmids or Semliki Forest virus (SFV).

Results. All prime-boost regimens elicited NS3-specific B and T cell responses in rhesus macaques, including CD8^{+} responses. SFV priming induced higher lymphoproliferation and longer Th1 memory responses. The use of IL-2– and IL-12–expressing vectors resulted in reduced Th2 and antibody responses, which led to increased Th1 skewing but not to an increase in the magnitude of the IFN-γ and CD8^{+} responses.

Conclusions. All strategies induced Th1 cellular responses to HCV NS3, with fine modulations depending on the different priming approaches. When they are developed for more HCV antigens, these strategies could be beneficial in therapeutic vaccine approaches.

Hepatitis C virus (HCV) is the major cause of chronic liver infection; it leads to severe liver damage, including cirrhosis and hepatocellular carcinoma [1]. Prophylactic and therapeutic approaches to prevent HCV hepatitis are urgently needed [2]. Although information regarding the immune correlates of protective immu-

nity is incomplete, current evidence points to the pivotal role played by HCV-specific cellular immune response in the control and clearance of HCV infection [3]. In particular, nonstructural protein (NS) 3–specific Th1 responses have been linked to viral clearance in humans [4, 5], and NS3-specific CD8^{+} responses have been described in the treatment-linked resolution of infection [6]. In chimpanzees, early viral clearance and protection from chronic infection have been associated with induction of interferon (IFN)–γ in the liver [7] and with envelope- and NS3-specific IFN-γ–secreting cells in the periphery [8].

To induce such immune responses, priming of the immune system is critical. Faulty priming that results in an inappropriate skewing of the immune system may be irreversible, and it may have dramatic adverse effects [9, 10]. T cell immunity can be induced against a variety of pathogens in different animal models by vaccine vec-

Table 1. Rates of seroconversion to nonstructural protein (NS) 3 after each immunization and after long-term follow-up (week 45).

Vaccine group	Antibody responders to NS3				
	After first prime	After second prime	After first adenovirus	After second adenovirus	Week 45
Group 1, DNA and adenovirus	0/3	1/3[a]	2/3	3/3	3/3
Group 2, DNA, IL, and adenovirus	0/4	0/4	1/4	4/4	1/4
Group 3, SFV and adenovirus	0/4	1/4[b]	1/4	2/4[c]	1/4[b]
Total	0/11	2/11	4/11	9/11	5/11

NOTE. Results are expressed as no. of macaques showing an antibody response to NS3/total no. of macaques in the group. IL, interleukin; SFV, Semliki Forest virus.

[a] Macaque Ri499.
[b] Macaque C056.
[c] Macaques C228 and Ri426 did not mount any detectable anti-NS3 antibody response.

tors, including DNA plasmids [8, 11–13] and recombinant viral vectors, such as Semliki Forest virus (SFV) [14, 15] and adenovirus [16]. Prime-boost immunization approaches with heterologous vectors are now being widely tested against different pathogens and are acceptable for human use [17]. We used adenovirus as a boosting agent because it infects a broad spectrum of human cells, including dendritic cells, which leads to efficient antigen presentation [18, 19] and therefore may enhance the immune responses induced by different priming vectors, such as DNA and SFV. The low-level immune responses elicited by DNA vaccines can also be influenced by combining them with cytokines, as has been demonstrated in the context of HIV in rhesus macaques [20–23]. We used plasmids encoding 2 interleukins (ILs) that favor the development of Th1 immune responses—IL-12 and IL-2 [22, 24]. Finally, the use of viral vaccine vectors such as SFV may have the advantage over plasmid DNA of mimicking a transient virus infection and, thus, recruiting innate antiviral responses [25, 26].

Although DNA, SFV, and adenoviral vectors have been evaluated in macaques as vaccine candidates for HIV, they have been the focus of only a small number of studies of HCV, which were limited to mice [27]. However, observations in mice can rarely be translated directly to humans, particularly those that concern Th1/Th2 response skewing. Besides humans, chimpanzees are the only species susceptible to chronic HCV infection; however, their use in research is highly restricted. Therefore, human clinical trials of HCV vaccine candidates may be guided by comparative immunogenicity studies in nonhuman primates, such as rhesus macaques. Although they are not susceptible to HCV infection, rhesus macaques are a suitable model for preclinical immunogenicity studies because they are phylogenetically and physiologically similar to humans [28]. Their well-characterized immune system is comparable to that of humans in terms of cytokine production and regulation [29]. HCV vaccine candidates based on recombinant viral proteins (core and, most recently, virus-like particles [VLPs]) have been tested for immu-

nogenicity in macaques and baboons [30, 31]. To date, there has been no knowledge of the responses induced in nonhuman primates by viral vectors encoding HCV antigens, and the results obtained in primates with vectors encoding other antigens, such as those for HIV, may not predict the responses obtained with HCV antigens that possess immunomodulatory functions [32–34]. We investigated the immunogenicity and fine modulation of B and T cell immune responses induced in nonhuman primates by comparing DNA and SFV priming in combination with adenovirus boosting. We used a clinically relevant HCV NS3 gene as a first screening step before incorporating other key HCV
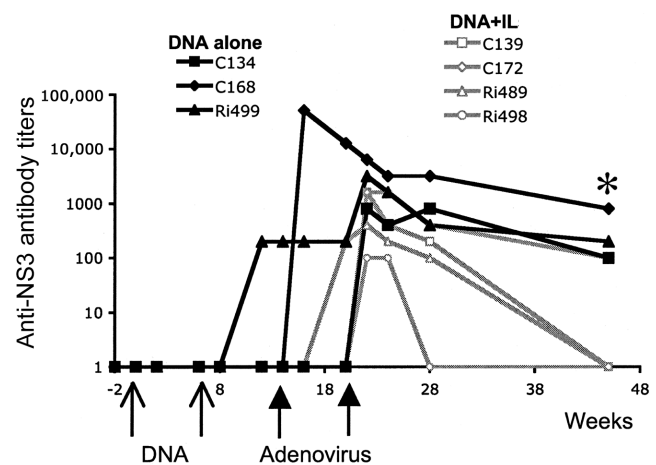
Figure 1. Anti–nonstructural protein (NS) 3 helicase antibody response in macaque groups primed with DNA and DNA plus cytokines. ELISA antibody titers are represented for each rhesus macaque in group 1, which were primed with DNA-NS3 alone (black lines and symbols) and of group 2, primed with DNA-NS3 plus interleukin (IL)–2 and IL-12 (gray lines and white symbols). White arrows represent the 2 priming injections; black arrows represent the 2 boosting injections. Results for group 3, primed with Semliki Forest virus and NS3, are not shown; only 2 of 4 macaques seroconverted. *Significant difference observed between the 2 groups (week 45, $P = .04$).
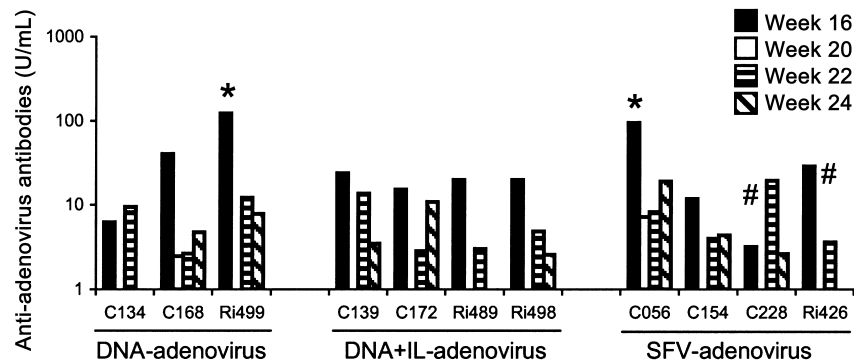
Figure 2. Anti-adenovirus antibody responses. Anti-adenovirus antibody titers are represented for all rhesus macaques at weeks 16, 20, 22, and 24. All macaques had undetectable anti-adenovirus antibody levels before the injection of adenovirus (data not shown). Immunizations with adenovirus–nonstructural protein (NS) 3 were performed at weeks 14 and 20. The immunization regimen for all macaques is indicated. SFV, Semliki Forest virus. *Two macaques (Ri499 and C056) seroconverted to NS3 after priming. #Two macaques (C228 and Ri426) did not elicit anti-NS3 antibodies at any time.

antigens, such as the structural proteins. These studies may be useful in the selection of vaccine candidates for efficacy testing.

MATERIALS AND METHODS

Macaques. The present study and the experimental procedures used in 12 naive rhesus macaques *(Macaca mulatta)* were approved by the institute's animal ethical and use committee and were performed in accordance with Dutch and international guidelines for the use of animals in science. Serum and peripheral blood mononuclear cells (PBMCs) were isolated from blood samples collected from sedated macaques at regular time points by use of aseptic techniques (Vacutainer; Becton Dick-

inson). Body weight, temperature, and hematologic and biochemical clinical values were monitored at regular intervals.

Peptides and recombinant NS3 protein. Seventy-eight 15-mer peptides with overlaps of 7 amino acids covering the NS3 region of the same HCV genotype 1b J strain [35] as that used for immunization were purchased from Clonestar Biotech. The NS3 helicase (aa 1193–1458) of HCV genotype 1a [36] was expressed in *Escherichia coli* and purified as described elsewhere [37].

NS3-expressing vaccine vectors. All immunogens used to immunize monkeys (DNA, SFV, and adenovirus) were based on the HCV genotype 1b J strain [35]. The DNA-NS3 plasmid
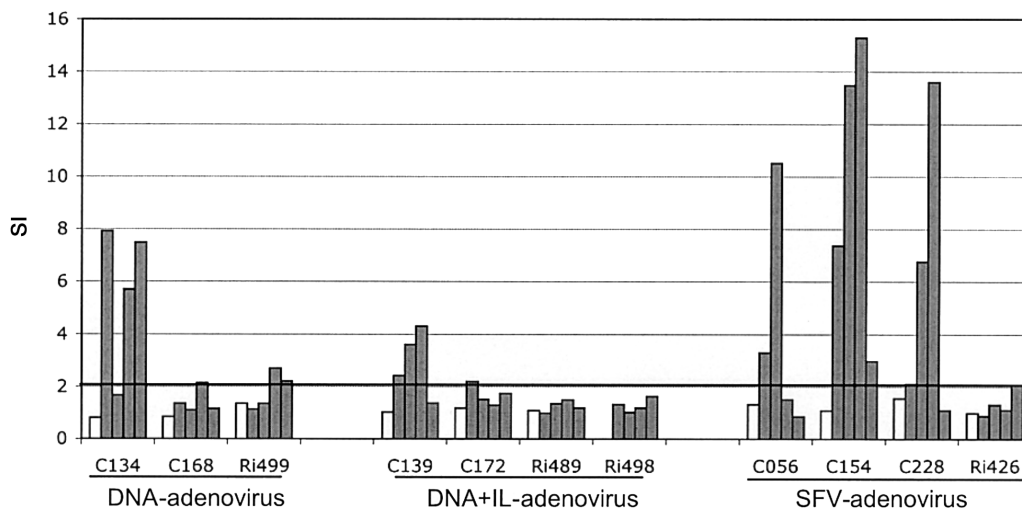
Figure 3. Lymphoproliferation to nonstructural protein (NS) 3 helicase protein. The individual stimulation indices (SIs) from each rhesus macaque from the 3 groups are shown. White bars indicate levels before immunization. Gray bars indicate levels after prime (week 8 or 12), after the first injection of NS3-adenovirus (week 16 or 20), after the second injection of NS3-adenovirus (week 22 or 24) (for these 3 time points, the better result for each macaque is shown), and week 45. Lymphoproliferation was considered to be positive when the SIs (counts per minute with antigen divided by background counts per minute with medium alone) was >2. The immunization regimen for all macaques is indicated. SFV, Semliki Forest virus.
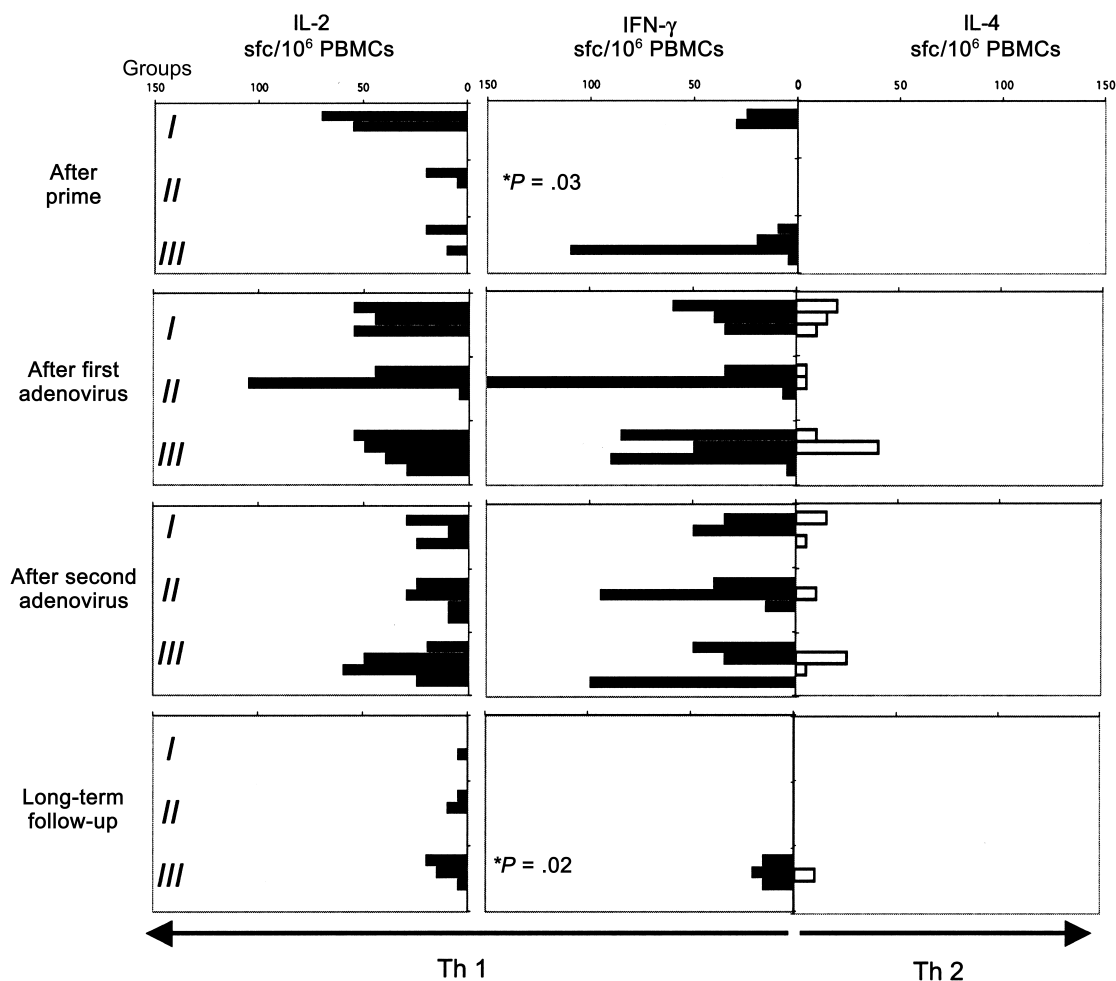
Figure 4. Individual balance of nonstructural protein (NS) 3 helicase–specific Th1 (interleukin [IL]–2 and interferon [IFN]–γ) and Th2 (IL-4) cytokine production by peripheral blood mononuclear cells (PBMCs). Cells secreting IL-2 *(left panels, black bars)*, IFN-γ *(middle panels, black bars)*, and IL-4 *(right panels, dashed bars)*, as tested by enzyme-linked immunospot assay, are shown for each macaque from each group (group 1, DNA priming; group 2, DNA plus IL priming; and group 3, Semliki Forest virus [SFV] priming) after prime boost (week 8 or 12), after the first injection of NS3-adenovirus (week 16 or 20), after the second injection of NS3-adenovirus (week 22 or 24) (the better result for each macaque is shown), and at week 45 (referred to as "long-term follow-up"). Results are expressed as mean no. of spot-forming cells in triplicate assays per 10^6 cells minus the cutoff (the mean no. of spot-forming cells obtained with the cells cultured in medium, also in triplicate assays, plus 2 SDs). Macaque identities are not indicated, but macaques are consistently represented for each group from top to bottom: group 1, C134, C168, and Ri499; group 2, C139, C172, Ri489, and Ri498; and group 3, C056, C154, C228, and Ri426. *Statistically significant difference with the other groups ($P < .05$, analysis of variance).

contains the NS3 gene (nt 3419–5311; aa 1027–1657), cloned into the high-expression gWIZ vector (Gene Therapy Systems) under the control of a modified cytomegalovirus promoter [38]. For SFV-NS3 particles, the NS3 fragment was inserted into an SFV expression vector. The packaging of RNA into recombinant SFV particles was facilitated by use of a 2-helper RNA system described elsewhere [39]. The production of NS3 was confirmed by in vitro transfection, pulse chase, and immunoprecipitation (data not shown). The NS3-expressing type 5 replication–defective adenovirus (NS3-adenovirus) has been described elsewhere [37]. HCV NS3-encoding DNA, SFV, and adenovirus constructs

elicited, alone or in prime-boost combinations, Th1 immune responses in mouse models [37, 38, 40, 41].

IL-encoding DNA plasmids. The human (h) IL-2/Ig plasmid pVRC-hIL2/Ig (DNA–IL-2), which encodes a fusion protein consisting of IL-2 and the Fc portion of IgG [21], was provided by Dr. Dan Barouch (Beth Israel Deaconess Medical Center, Boston, MA). In rhesus macaques, this markedly augments DNA vaccine-elicited HIV-1 and simian immunodeficiency virus (SIV)–specific immune responses [21]. The plasmid pNDI–IL-12, which encodes rhesus IL-12β (DNA–IL-12; provided by Francois Villinger, Emory University, Atlanta, GA),
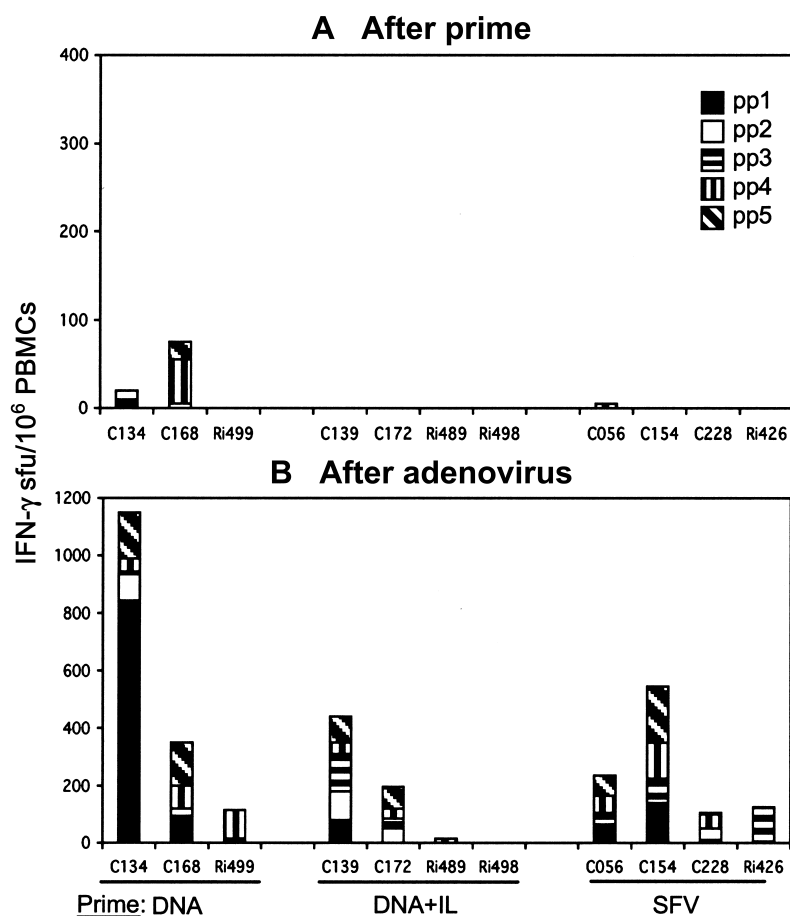
Figure 5. Individual cumulative nonstructural protein (NS) 3 peptide pool–specific interferon (IFN)–γ production by peripheral blood mononuclear cells (PBMCs). Production of IFN-γ to NS3 peptide pools (pp) 1–5, as tested by enzyme-linked immunospot assay, are shown for each macaque from each group after prime boost (*A,* week 8 or 12) and after the injection of NS3-adenovirus (*B,* week 16, 20, 22, or 24) (the better result for each macaque is represented). Results are expressed as mean no. of spot-forming cells in triplicate assays per 10^6 cells minus the cutoff (the mean no. of spot-forming cells obtained with the cells cultured in medium, also in triplicate assays, plus 2 SDs). SFV, Semliki Forest virus.

has been reported to enhance DNA vaccine-induced protection of rhesus macaques against SIV challenge [42]. All plasmid DNAs were extracted and purified by use of Qiagen EndoFree Plasmid Kits (Qiagen).

Immunizations. The 3 prime-boost vaccine combinations were tested in 3 groups of 4 macaques each. The first group received the DNA prime and adenovirus boost, the second group received the DNA plus cytokine-encoding plasmids prime and adenovirus boost, and the third group received the SFV prime and adenovirus boost. The priming consisted of 2 injections at weeks 0 and 6, and the NS3-adenovirus boosters were administered at weeks 14 and 20. A prime is the first time an antigen is encountered, but, for brevity, the first and second immunizations (DNA or SFV) will be referred to as "prime," and the third and fourth injections (adenovirus) will be referred to as "boost." For each DNA immunization, 1 mg of DNA-NS3 suspended in saline buffer was equally divided intramuscularly and intradermally. For group 2, 1 mg of DNA–IL-12 was

coadministered with DNA-NS3, and 1 mg of DNA–IL-2 was administered 2 days later at the same sites. For SFV-NS3 immunization, 5×10^8 pfu dissolved in saline were injected subcutaneously. All macaques were boosted subcutaneously with 5×10^9 pfu NS3-adenovirus at week 14 and with 5×10^{10} pfu at week 20. No adverse effects or lesions associated with the inoculations were found. One macaque from the first group died of an unrelated health problem at week 6, reducing group 1 (DNA and adenovirus) to 3 macaques.

Humoral immune responses. Quantification of anti-adenovirus antibodies was performed in serum by an independent hospital laboratory (Erasmus MC—Virology), by use of a quantitative ELISA (SERION adenovirus IgG/IgA) that detects antibodies to the 8 most prevalent serotype-specific epitopes. HCV-NS3–specific antibodies were detected in serum by ELISA for which microtiter plates were coated with NS3 helicase recombinant protein (0.5 μg/mL). Serum samples were screened at a dilution of 1:100. Positive samples were serially diluted to
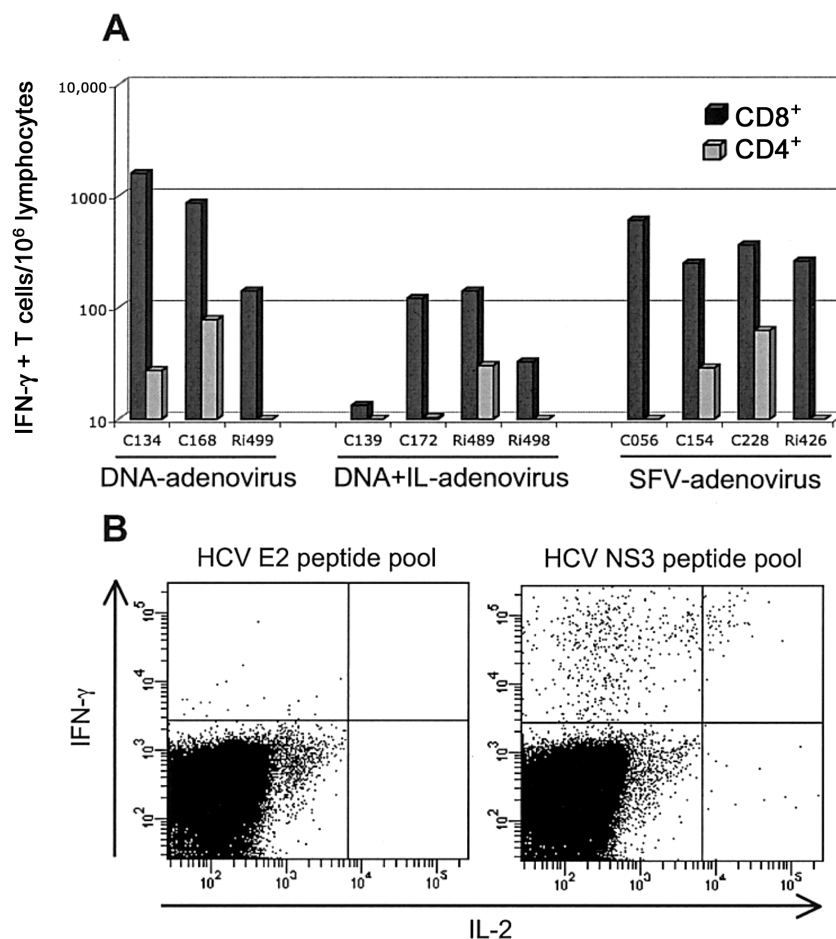
Figure 6. Individual production of interferon (IFN)–γ to nonstructural protein (NS) 3 peptide pools (pp) by CD4+ and CD8+ cells. *A,* NS3 peptide–specific IFN-γ–positive CD4+ *(gray bars)* and CD8+ *(black bars)* responses were analyzed in all rhesus macaques by an intracellular staining assay after injections of adenovirus (week 20 or 24; the better result for each macaque is shown). Results are expressed as corrected no. of NS3-specific IFN-γ–positive CD4+ or CD8+ cells per 10^6 lymphocytes (no. of IFN-γ–positive CD4+ or CD8+ cells with NS3 peptide pool minus the no. of IFN-γ–positive CD4+ or CD8+ cells with an irrelevant hepatitis C virus [HCV] E2 peptide pool). *B,* As an example, the flow-cytometric dot plots of the IFN-γ– and IL-2–producing CD8+ cells from macaque C134 stimulated with the irrelevant HCV E2 peptide pool *(left panel,* negative control) and with the HCV NS3 peptide pool *(right panel)* are represented. Lymphocytes are gated for CD3+ and CD8+ surface markers. PBMC, peripheral blood mononuclear cells.

obtain end-point titers. Detection was performed with peroxidase-conjugated Affinipure goat anti–human IgG (Jackson Immunoresearch), diluted to 1:10,000. The cutoff was determined as the mean value obtained with serum from 3 naive blood donors +3 SDs. Serum was considered to be positive when the absorbance was equal to or superior to this cutoff.

Cellular immune responses. Lymphoproliferation was measured by ^{3}H-thimidine incorporation, as described elsewhere [34]. Quantification of specific cytokine-secreting cells was performed by IFN-γ, IL-2, and IL-4 enzyme-linked immunospot (ELISPOT) assays, according to the manufacturer's instructions (U-Cytech), by use of concanavalin A (ConA; 5 μg/mL), NS3 helicase protein (4 μg/mL), peptides covering NS3 or HIV-1$_{SF2}$ gag p24 (4 μg/mL; provided by the late Kathelyn Steimer, Chiron, Emeryville, CA) or medium alone. Results are expressed as the mean number of spot-forming cells per 10^6 cells from

triplicate assays minus the cutoff (mean number of spot-forming cells plus 2 SDs obtained with triplicate medium assay).

Phenotyping by intracellular cytokine staining (ICS) assay. The phenotype of responding T cells was analyzed by use of ICS assay, as described elsewhere [43], by stimulating PBMCs with ConA, NS3, or E2 peptide pools (5 μg/mL) or with medium alone and by staining with fluorescein isothiocyanate–labeled anti-CD3, peridinin-chlorophyll-protein complex–labeled anti-CD8, and allophycocyanin-labeled anti–IFN-γ antibodies (BD Pharmingen). Results are expressed as the corrected number of specific IFN-γ–positive CD4+ or CD8+ cells per 10^6 lymphocytes, according to the method of Barouch et al. [22]: numbers of IFN-γ–positive CD4+ or CD8+ cells with the NS3 peptide pool minus the numbers of IFN-γ–positive CD4+ or CD8+ cells with the irrelevant E2 peptide pool.

Statistical analysis. Statistical analysis was performed by

analysis of variance and *t* tests, with 2-tailed *P* values calculated by exact methods. *P* < .05 was considered to be significant.

RESULTS

Influence of priming on the antibody responses. The priming injections were poor inducers of antibody responses in rhesus macaques (table 1). After 2 adenovirus boosts, all 7 macaques primed with DNA seroconverted, whereas this was true for only 2 of 4 SFV-NS3–primed macaques. In the macaques that received only DNA, 2 of 3 had seroconverted after the first adenovirus injection, as did 1 of 4 in the cytokine-adjuvanted group. Analysis of the antibody titers in these DNA-primed groups suggested that the macaques primed with NS3 plus plasmids encoding IL-2 and IL-12 had lower antibody titers (range, 100–1600), compared with the macaques primed with DNA-NS3 alone (range, 800–51,200) (figure 1). The antibody response was higher in the DNA-primed group at week 45 (*P* = .04).

Transient anti-adenovirus humoral responses were induced in all macaques, peaking 2 weeks after the first adenovirus injection (3.19–122.0 U/mL at week 16; figure 2) and decreasing to undetectable levels at week 28 (data not shown). The 3 priming vectors did not influence the adenovirus-specific humoral response. Two macaques that seroconverted to NS3 after priming (Ri499 and C056) mounted higher responses to the adenoviral vector (122 and 94 U/mL, respectively) than did the others (maximum, 40.3 U/mL), which suggests that NS3-specific T helper cells elicited by the priming immunizations provided T cell help to the adenovirus-specific B cells. Except for these cases, there was no correlation between the anti-NS3 and the anti-adenovirus antibody titers. The 2 SFV-NS3–primed macaques that did not seroconvert to NS3 (C228 and Ri426) had anti-adenovirus antibody responses (19.4 and 28.8 U/mL, respectively; figure 2), which confirms that the absence of NS3 seroconversion was not due to an improper injection of NS3-adenovirus. Owing to the likelihood of preexisting adenovirus immune responses limiting the second boost, we elected to use a 10-fold-higher second dose. Despite this, the second administration of NS3-adenovirus elicited a lower response (from 0 to 19.4 at weeks 22 and 24; figure 2).

Impact of priming on lymphoproliferative responses. Both combinations involving DNA-NS3 priming were poor inducers of lymphoproliferative responses, compared with the SFV-primed macaques (*P* = .04; figure 3). Only 2 of 7 DNA-primed macaques (C134 and C139) showed proliferation (stimulation indices [SIs] peaking at 7.9 and 4.3, respectively), whereas the other macaques had a marginal or no response (SI ≤ 2.7). In contrast, 3 of 4 macaques immunized with SFV-adenovirus developed higher NS3-specific lymphoproliferative responses, with SIs of 6.8–15.3 (*P* = .04). However, this response became undetectable at week 45 in almost all macaques (figure 3).

Differential induction of Th1/Th2 cytokine secretion. We counted the NS3 protein–specific IL-2–, IFN-γ–, and IL-4–secreting cells (figure 4). Although we did not separate CD8^{+} cells from the PBMCs, the cells responding to proteins in the ELISPOT assay most likely were CD4^{+} cells [44]. All 3 vaccine regimens induced IL-2 responses to NS3, with comparable intensity and kinetics between groups (figure 4). Most responses were obtained after the first injection of NS3-adenovirus (range, 10–105 sfc/10[6] cells) and were not boosted by the second injection of NS3-adenovirus. Low numbers of IL-2–secreting cells were still detectable 25 weeks after the last injection in 6 macaques, 3 of which had been primed with SFV.

The 3 vaccine regimens were also able to induce NS3-specific IFN-γ–secreting T cells that largely exceeded the IL-4 responses (figure 4). Surprisingly, the use of cytokine-encoding plasmids blocked IFN-γ production after the priming injection (*P* = .03). After the first injection of NS3-adenovirus, IFN-γ responses in all 3 groups were equivalent (range, 15–150 sfc/10[6] cells) and were not further increased by the second adenovirus boost (figure 4). IFN-γ responses became undetectable by week 28 in all 7 DNA-primed macaques, whereas SFV priming elicited IFN-γ responses in macaques up to week 45 (*P* = .02). NS3-specific IL-4–producing cells were induced after the injections of NS3-adenovirus in only 6 of 11 macaques and were low in all of them (range, 5–40 sfc/10[6] cells; figure 4). In particular, DNA with IL-2 and IL-12 reduced the IL-4 responses to almost undetectable levels (to <10 sfc/10[6] cells). Taken together, these results demonstrate that the 3 vaccine regimens were able to induce NS3 protein–specific Th1 responses, but priming with IL-2 and IL-12 did not increase or prolong the IFN-γ responses.

Induction of IFN-γ responses to NS3 peptide pools. The induction of NS3-specific CD8^{+} responses was a primary objective of our HCV vaccine experiments, because of the observed benefit that cytotoxic T lymphocyte responses give in the control of HCV infection. As a first screening, we undertook an analysis of IFN-γ production in response to 5 NS3 peptide pools (pp) covering the entire NS3 protein: pp1 covered aa 1028–1154, pp2 covered aa 1148–1274, pp3 covered aa 1268–1402, pp4 covered aa 1395–1530, and pp5 covered aa 1524–1644 (pp1 and pp5 covered regions that were not included in the NS3 helicase recombinant protein). Before the adenovirus injections, low IFN-γ production specific to pp1, pp4, and pp5 was induced in macaques C134 and C168, which had been primed with DNA-NS3 (20 and 75 sfc/10[6] cells, respectively; figure 5*A*). After the injections of NS3-adenovirus, 9 of 11 macaques in all groups had strong IFN-γ responses to ≥1 peptide pool, with cumulative responses reaching 1150 sfc/10[6] cells. At week 28, most peptide pool–specific responses were <45 sfc/10[6] cells in all groups (data not shown).

Phenotype of the NS3 peptide–specific response. We investigated the phenotype of the NS3 peptide–specific IFN-γ–

producing T cells using the ICS assay. Samples obtained after adenovirus infection were analyzed by use of a single NS3 peptide pool including pp1–pp5. Our results demonstrated that high peptide-specific IFN-γ production was due to CD8+ cells, with NS3-specific IFN-γ–positive CD8+ cell counts of 13–1588 cells/10^6 PBMCs (figure 6). Significantly lower responses were observed in the IL-2 and IL-12 group, compared with the 2 other groups ($P = .02$).

DISCUSSION

We evaluated the immunogenicity of 3 vaccine regimens aimed at inducing Th1 and CD8+ immune responses to the HCV NS3 antigen in a nonhuman primate model, the rhesus macaque. We compared a DNA prime, DNA plus IL-2 and IL-12 prime, and an SFV prime, all of which were combined with an adenovirus boost. Although similar studies have been performed with HIV, these vaccine combinations are unique in the field of HCV. Our results showed that all vaccine regimens were able to induce B cell, Th1, and CD8+ cell responses, with fine differences.

The priming agents DNA and SFV alone were not able to induce good antibody responses in rhesus macaques, whereas the blocking with the adenovirus was highly efficient, as has been demonstrated elsewhere [45]. Similarly, B cell responses can be elicited with HIV DNA or SFV immunization alone [46, 47]; however, viral vector boosting is beneficial for reaching significant titers [48, 49]. A lower and short-lived B cell response was obtained with the IL-2 and IL-12 priming, as was expected, given that the induction of a Th1 bias should have counteracted and limited the Th2 response [50]. Therefore, the cytokine priming had a long-lasting effect, because the first and second adenovirus immunizations occurred 8 and 16 weeks, respectively, after the last cytokine injection, which emphasizes the importance of the "imprinting" left during the first encounter of the immune system with an antigen.

Similar to the B cell response, most NS3-specific cellular responses were observed after adenovirus boosting. As with HIV constructs, plasmid DNA alone does not elicit T cell responses of sufficient magnitude in primates [34]. We observed that the second injection of adenovirus did not increase the NS3-specific cellular immune responses, even while it elicited a reduced anti-adenovirus antibody response. This suggests that the 10-fold-higher dose administered at the second injection was neutralized by the antivector immune response. Indeed, the impact of preexisting immune memory on vaccine viral vectors and especially adenovirus has previously been documented with other vaccines, including HIV vaccines, in primates [51, 52]. Although it seems that the second injection of adenovirus was beneficial for the humoral response, this still could have been due to a delayed effect of the first injection of adenovirus. In other studies, longer intervals of 12 or 24 weeks allowed a second injection of adenovirus, which was shown to have a boosting effect on both humoral and cellular responses in macaques and suggests that the second injection of adenovirus has a boosting effect only after the specific cellular responses have undergone the contraction phase [53, 54]. The 2 injections of adenovirus in our study were probably too close to each other.

As expected, and similar to results previously observed with HIV vaccines for which IL-4 production was measured [34], the 3 vaccine strategies induced Th1-biased immune responses in primates, with IFN-γ responses largely exceeding the IL-4 responses, and included the desired NS3-specific CD8+ responses. However, the cytokines increased the skewing of the CD4+ response toward Th1 but did not increase the intensity of the IFN-γ and IL-2 responses and even decreased the CD8+ response. This is surprising, because both the IL-2 and IL-12 plasmids used in the present study have demonstrated their potential to enhance and improve the efficacy of several T cell–based HIV vaccines in macaques [21, 22]. The IL-12 dose released in the microenvironment of antigen-presenting cells (APCs) and T cells seems to be crucial. In particular, a high dose might block the function of APCs and reduce the immunogenicity of the coinjected antigen, as has been described in mice [55, 56]. When DNA is used, the amount of cytokine released locally should be low. However, because we did not compare different doses, we cannot exclude the possibility that 1 mg of IL-12–encoding DNA produced too much IL-12 locally and thus reduced the immunogenicity of DNA and NS3. The observations that no IFN-γ was detected after the priming injections that included cytokines, in contrast with the 2 other priming strategies ($P = .03$), and that the CD8+ responses were lower ($P = .02$) point in this direction. The antigen itself may influence the quality of the immune responses, and this effect might be dominant over the cytokine microenvironment, as was shown recently for HIV antigens [34]. In vitro, HCV NS3 has been shown to induce IL-10 production by monocytes and dendritic cells while reducing IL-2 production and differentiation in the latter. Notably, this could be reversed by use of anti–IL-10 antibodies but not by the addition of IL-12 [33]. This property could account for the differences observed between HIV vaccines and our HCV NS3–based vaccine.

The development of prophylactic and therapeutic vaccine strategies against HCV infection cannot be limited to studies in mice, because there is a big gap between the results of mice and chimpanzee studies and those of human clinical trials. Although a high number of vaccine candidates have been developed and tested in mice [57], very few have reached the preclinical phase and have been tested in naive chimpanzees [8, 58, 59]. The use of nonhuman primates to investigate the immunogenicity of HCV vaccine candidates is a valid option, and it may help in the selection of optimal vaccine candidates or adjuvants and allow a detailed analysis of several aspects of the immune responses. Both HCV vaccine candidates tested pre-

viously in nonhuman primates (macaques and baboons) were based on subunit proteins (core and VLPs, respectively) and did not involve NS3 [30, 31]. Our vaccine regimens elicited HCV-specific responses that were comparable in intensity to those of responses observed in baboons immunized with VLPs, and these responses mimic some of those observed in patients recovering from HCV infection [5]. In conclusion, we have demonstrated the utility of clinically relevant DNA and SFV prime–adenovirus boost vaccine strategies for the induction of HCV-specific Th1 and CD8^{+} cellular responses in primates and have shown the effect of priming on the quality of the ultimate vaccine-induced response.

Acknowledgments

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