

# Hepatitis C virus non-structural protein 3-specific cellular immune responses following single or combined immunization with DNA or recombinant Semliki Forest virus particles

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The capacity of recombinant Semliki Forest virus particles (rSFV) expressing the hepatitis C virus non-structural protein 3 (NS3) to induce, in comparison or in combination with an NS3-expressing plasmid, specific cellular and humoral immune responses in murine models was evaluated. *In vitro* studies indicated that both types of vaccine expressed the expected size protein, albeit with different efficacies. The use of mice transgenic for the human HLA-A2.1 molecule indicated that the rSFV-expressed NS3 protein induces, as shown previously for an NS3 DNA vaccine, NS3-specific cytotoxic lymphocytes (CTLs) targeted at one dominant HLA-A2 epitope described in infected patients. All DNA/rSFV vaccine combinations evaluated induced specific CTLs, which were detectable for up to 31 weeks after the first injection. Overall, less than 1 log difference was observed in terms of the vigour of the bulk CTL response induced and the CTL precursor frequency between all vaccines (ranging from  $1:2.6 \times 10^5$  to  $1:1 \times 10^6$ ). Anti-NS3 antibodies could only be detected following a combined vaccine regimen in non-transgenic BALB/c mice. In conclusion, rSFV particles expressing NS3 are capable of inducing NS3-specific cellular immune responses targeted at a major HLA-A2 epitope. Such responses were comparable to those obtained with a DNA-based NS3 vaccine, whether in the context of single or combined regimens.

## Introduction

Hepatitis C virus (HCV), the major causative agent of non-A, non-B hepatitis (Alter *et al.*, 1978; Choo *et al.*, 1990), infects an estimated 170 million people worldwide (Cohen, 1999). HCV was first isolated in 1989 (Choo *et al.*, 1989) and belongs to the family *Flaviviridae*. Its genome is composed of a positive-sense, single-stranded RNA encoding a polyprotein comprising structural (core and envelope glycoproteins E1 and E2) and non-structural (NS2, NS3a/b, NS4a/b and NS5a/b) proteins. In 50% (or more) of cases, acute HCV infections result in chronic hepatitis, possibly leading towards cirrhosis and hepatocellular carcinoma (Hoofnagle, 1997; Saito *et al.*, 1990). The mechanisms ruling out HCV-associated chronicity are still poorly understood, but mounting evidence suggesting that

HCV infects cells of the immune system indicates that such extra-hepatic reservoirs are likely to play a key role in the establishment of virus persistence (Bain *et al.*, 2001; Kanto *et al.*, 1999; Lerat *et al.*, 1996).

Several comparative studies performed in chronically infected patients or patients with a self-limited infection have shown that a specific cellular immune response (CD4<sup>+</sup>- or CD8<sup>+</sup>-mediated) is mounted quickly by the host following virus exposure (6–8 weeks after exposure). This response is, nonetheless, not sustained very often and changes in its nature with time (Prezzi *et al.*, 2001; Botarelli *et al.*, 1993; Diepolder *et al.*, 1995, 1997; Gerlach *et al.*, 1999; Lamonaca *et al.*, 1999; Lechner *et al.*, 2000a, b; Nelson *et al.*, 1997; Rehmann *et al.*, 1996). Such a response is typically multi-specific in chronic carriers but is inefficient and has to be enhanced and sustained to lead to virus elimination (Poynard *et al.*, 1998). Among the HCV antigens involved in eliciting host immune responses, the NS3 protein, a viral serine protease and helicase

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(Grakoui *et al.*, 1993), seems to play a key role in virus clearance. It contains an immunodominant CD4<sup>+</sup> T helper epitope, which has been associated specifically with the control of HCV infection in self-limited patients, as well as numerous cytotoxic T lymphocyte (CTL) epitopes (Battegay *et al.*, 1995; Cerny *et al.*, 1995; Diepolder *et al.*, 1997; Kurokohchi *et al.*, 1996). Thus, NS3 represents a potential vaccine candidate able to induce both CD4<sup>+</sup>- and CD8<sup>+</sup>-mediated protective immune responses.

Today, several vaccine strategies based on peptides or protein subunits (Choo *et al.*, 1994; Lopez-Dias de Cerio *et al.*, 1999), recombinant vaccinia virus-vectored vaccines (Shirai *et al.*, 1992), adenovirus- or baculovirus-derived vectors (Baumert *et al.*, 1999; Makimura *et al.*, 1996) and naked DNA-based vaccines (Brinster & Inchauspé, 2001) are pursued. One approach that has not yet been examined extensively is the use of the Semliki Forest virus (SFV) system described recently. SFV belongs to the genus *Alphavirus* of the family *Togaviridae*, which have a wide host range and replicate in a variety of different species, ranging from mammals to insects. Their genome is able to self-replicate as it encodes its own replicase (nsP1 to nsP4) and replication results in a high level of expression of the viral proteins in host cells (Schlesinger & Schlesinger, 1996). In vaccine development, the SFV system was employed first as a DNA- or an RNA-derived vaccine (Ying *et al.*, 1999), where the SFV structural genes were replaced by a foreign gene. We have evaluated previously an SFV-derived plasmid expressing the NS3 protein in comparison with the more classical DNA-based vaccines (Brinster *et al.*, 2001). In contrast to this approach, the use of recombinant SFV (rSFV) particles should permit an enhanced transduction of cells compared with DNA- or RNA-based vaccines (Leitner *et al.*, 1999). These rSFV particles infect host cells but do not replicate: only the foreign gene introduced is expressed at high levels. This approach has been shown to enhance humoral and cellular immune responses in mice and monkeys in different virus models, such as influenza virus, louping ill virus (LIV), human immunodeficiency virus (HIV) and human papillomavirus (HPV) (Berglund *et al.*, 1999; Brand *et al.*, 1998; Daemen *et al.*, 2000; Fleeton *et al.*, 2000; Reddy *et al.*, 1999). While DNA immunization has been shown in a large variety of models to induce long-lasting specific CTLs in mice, its efficacy in primates and humans remains quite controversial (Barouch *et al.*, 2000; Calarota & Wahren, 2001; Lodmell *et al.*, 1998; Polack *et al.*, 2000). Remarkable enhancement of cellular immune responses has, nonetheless, been demonstrated recently in both mice and primates when DNA-based vaccines were used in combined regimens, either with recombinant antigens or recombinant viruses (Amara *et al.*, 2001; Pancholi *et al.*, 2000; Robinson *et al.*, 1999).

The focus of our study was to investigate the capacity of SFV-derived particles to induce NS3-specific CD8<sup>+</sup>-mediated responses in comparison or in combination with a naked DNA-based vaccine. For this purpose, we used a recently developed

HLA-A2.1 transgenic mouse model devoid of murine MHC class I molecules in order to document the induction of NS3-specific immune responses targeted at naturally occurring epitopes (Pascolo *et al.*, 1997).

## Methods

■ **Mice.** C57BL/6 mice transgenic for the HLA-A2.1 (A0201) monochain and deficient for both *H-2D<sup>b</sup>* epitopes and murine  $\beta$ 2-microglobulin have been described by Pascolo *et al.* (1997). Female 6- to 8-week-old C57BL/6 transgenic mice as well as female BALB/c non-transgenic mice (*H-2<sup>d</sup>*), purchased from Iffa Credo, were used for our experiments. Mice were housed in appropriate animal care facilities and handled following international guidelines required for animal experiments.

■ **Recombinant expression vectors.** The NS3 gene of HCV strain H (Inchauspé *et al.*, 1991) was inserted into the commercial pCI vector (Promega) to create pCI.NS3, the expression of which is governed by the cytomegalovirus (CMV) promoter (Brinster *et al.*, 2001).

The SFV particles recombinant for the HCV NS3 protein (rSFV.NS3) were also generated to include the NS3 gene sequence of HCV strain H. Briefly, packaging of recombinant RNAs expressing NS3 was performed according to procedures described previously (Berglund *et al.*, 1998; Smerdou & Liljeström, 1999). Two helper SFV RNAs expressing the HCV NS3 protein and the SFV envelope proteins, respectively, were co-transfected into BHK-21 cells together with the HCV recombinant RNAs. Titration of infectious units (IU) by immunofluorescence was performed as described elsewhere (Liljeström & Garoff, 1994).

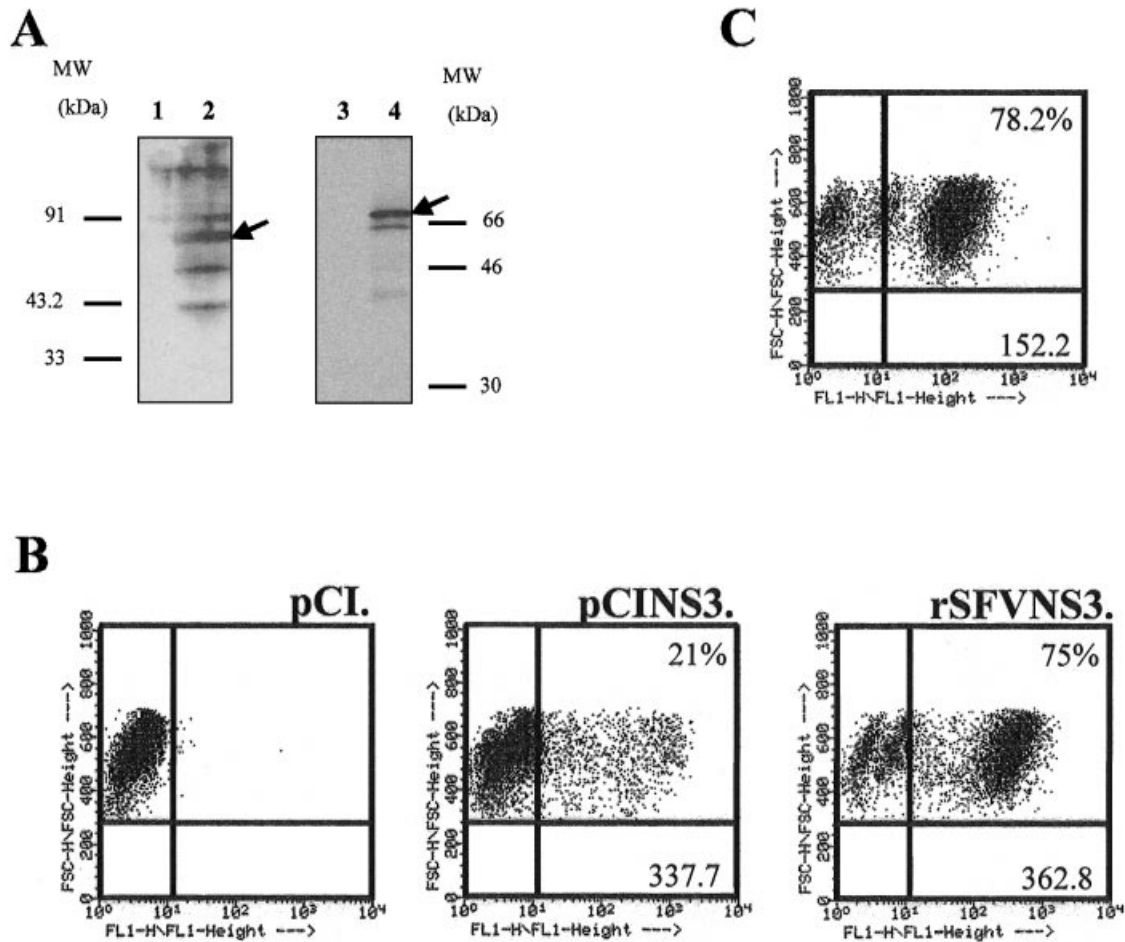
■ **In vitro studies.** Transient transfections of NIH 3T3 or BHK-21 cells were performed, as described previously, using 1  $\mu$ g DNA and corresponding volumes of Reagent plus and Lipofectamine plus (Life Technologies). Cells were incubated for 3 h at 37 °C in transfection medium and complete medium was then added.

Infections with rSFV.NS3 were performed using 80–100% confluent cells grown on 35 mm diameter plates. The cells were washed with PBS and the particles diluted in Glasgow's MEM containing 0.2% BSA, 2 mM L-glutamine and 20 mM HEPES buffer (Life Technologies). Viruses were applied at an m.o.i. of 10 and cells were incubated for 60–90 min at 37 °C. The virus inoculum was then removed and complete medium was added.

At 24 h post-transfection or -infection, cells were harvested for analysis of antigenic expression by flow cytometry and Western blotting. For Western blot studies, infected cells were lysed for 30 min at 4 °C with 50 mM Tris-HCl (pH 8), 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA and 10  $\mu$ g/ml aprotinin. Lysates were passed through a needle (0.5  $\times$  16 mm) and then clarified by centrifugation. Supernatants were loaded directly onto gels and resolved by SDS-PAGE. Western blot analysis was performed using anti-NS3 ascites fluid (33E8H10D4B8H4, a gift from Biomérieux, France) as the primary antibody. The secondary antibody was a goat anti-mouse immunoglobulin (Ig) monoclonal antibody (mAb) coupled to horseradish peroxidase (Caltag). Signals were revealed using the commercial ECL kit (Amersham-Pharmacia).

For flow cytometry, transfected or infected cells were fixed with 70% ethanol and stained with the anti-NS3 ascites or the anti-SFV replicase mAb (courtesy of M. Chen, Karolinska Institute, Sweden). A goat mAb directed at murine immunoglobulins and conjugated to FITC was used as a secondary antibody (Dako). Flow cytometry analysis was performed using a FACscan (Becton Dickinson).

■ **Proteins and synthetic peptides.** All synthetic peptides used in our experiments were purchased from Neosystem. Peptides were



**Fig. 1.** *In vitro* analysis of NS3 expression following transient transfection or infection of BHK-21 cells. Murine fibroblasts and BHK-21 cells were either transfected or infected with pCI.NS3 or rSFV.NS3 and the expression analysed by (A) Western blotting or (B, C) flow cytometry, as detailed in Methods. (A) Lane 1, pCI-transfected cells (mock); lane 2, pCI.NS3-transfected cells; lane 3, rSFV.LacZ-infected cells (mock); lane 4, rSFV.NS3-infected cells. The NS3 protein is indicated (70 kDa) by an arrow. Molecular mass (MW) markers are indicated on the sides of the gels. (B) Results of NS3-specific staining at either 24 h post-transfection or 24 h post-infection, respectively. (C) The SFV replicase protein expression observed 24 h post-infection. For all dot plots, the MFI value is indicated in the lower right quadrant and the percentage of transfection or infection is indicated in the upper right quadrant. Data are from a representative experiment.

dissolved in DMSO (20  $\mu$ l/mg) and diluted in distilled water to a final concentration of 1 mM. All HLA-A2.1-restricted CTL peptides were derived from the NS3 sequence of HCV strain H, except for one that was derived from a 1b genotype isolate (Kurokohchi *et al.*, 1996). The sequences of the peptides used were as follows: CINGVCWTV (aa 1073–1081), KLVALGINAV (aa 1406–1415), LLCPTGHAV (aa 1169–1177), TGSPIITYSTY (aa 1287–1296) and TGAPVITYSTY (aa 1287–1296, 1b genotype), referred to as CIN, KLV, LLC, TGS and TGA, respectively.

**Immunizations.** All DNA preparations were generated using Qiagen purification columns (Qiagen). Intraepidermal immunizations were performed using a helium-powered Accell instrument (Powderject). Mice were immunized with two shots, each containing 2.5  $\mu$ g DNA, as described previously (Nakano *et al.*, 1997).

rSFV particles were diluted in PBS and injected intravenously (into the tail veins) with  $10^6$  p.f.u. of virus per mouse. A total of four injections was performed at 2 or 3 week intervals. For combined injections, two

injections of pCI.NS3 or rSFV.NS3 were performed at 2 or 3 week intervals followed by two boost immunizations of either rSFV.NS3 or pCI.NS3 at 2 or 3 week intervals.

#### CTL analysis

**Bulk CTL assay.** Conditions were as described previously (Brinster *et al.*, 2001). Briefly, spleen cells from individual mice were stimulated for 5 days with 5  $\mu$ M peptide and then restimulated on day 5 using irradiated syngeneic splenocytes (30 Gy) pulsed with the selected peptide (5  $\mu$ M) in the presence of 5 U/ml murine recombinant interleukin (IL)-2 (PedroTech EC). On day 7, the stimulated cells were used as effector cells in a standard <sup>51</sup>Cr-release assay against EL-4S3<sup>-</sup>Rob HHD target cells (Pascolo *et al.*, 1997), either pulsed or not with 10  $\mu$ M of the selected peptide. Effector and target cells were also used at different ratios. After a 4 h incubation, <sup>51</sup>Cr release was measured using a  $\gamma$ -Cobra II counter (Packard). Spontaneous and maximum release were determined from wells containing either medium alone or lysis buffer (1 N HCl). Specific cytotoxicity was calculated using the following

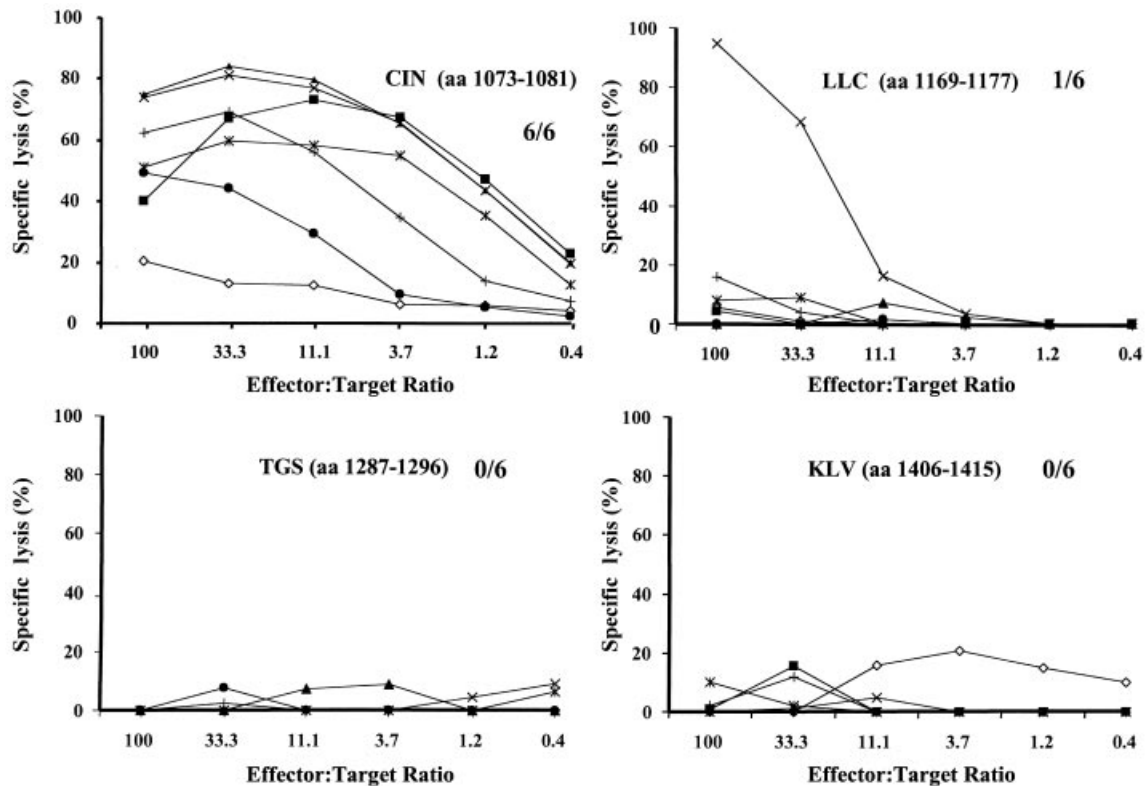


Fig. 2. Induction of anti-NS3-specific CTLs following vaccination with rSFV.NS3 particles. HLA-A2.1 mice were immunized intravenously two times at 2 week intervals with rSFV.NS3 particles. Spleen cells were collected 2 weeks after the final injection and cultured in the presence of each peptide tested. Bulk CTL analysis was performed as detailed in Methods. Mock-vaccinated mice ( $\diamond$ ) are represented. All other mice immunized are represented with black symbols. The number of mice responding out of the number of mice tested is indicated in the upper right corner of each graph.

formula: (release in assay – spontaneous release / maximum release – spontaneous release)  $\times$  100. For each effector:target (E:T) ratio, data are expressed as the mean of duplicate results. In some cases, lysis observed at high E:T ratios in the absence of peptide was unexpectedly high, which resulted in a low level of specific lysis for these E:T ratios compared with that observed for low E:T ratios.

**Limiting dilution assay to determine CTL frequency.** Such assays have been described previously (Brinster *et al.*, 2001). Briefly, immune splenocytes were mixed with the CTL peptide (5  $\mu$ M) and various concentrations of irradiated syngeneic splenocytes (30 Gy) in order to obtain  $0.8 \times 10^6$  total cells per well. After 5 days, cells were restimulated using irradiated syngeneic splenocytes pulsed with the selected peptide (5  $\mu$ M) in the presence of 5 U/ml murine recombinant IL-2. Seven days after the initial culture, a standard  $^{51}\text{Cr}$ -release assay was performed on each individual well. Responses for individual wells were considered to be positive when peptide-specific lysis was greater than 15%. Frequencies were calculated using  $\chi^2$  analysis. CTLs were calculated on the regression curve by interpolating the number of responder cells required to give 37% negative cultures.

■ **Measurement of humoral immune response.** Anti-NS3 antibodies in the sera of immunized mice were measured with a specific ELISA test, as described previously, using a recombinant NS3 protein (aa 1192–1457) (Brinster *et al.*, 2001).

■ **Titration of IgG antibodies specific for SFV particles.** Sucrose gradient-purified SFV4 particles were adsorbed passively overnight at 4 °C onto the wells of a microtitre plate (F96 Maxisorp, Nunc

Immunoplates) at a concentration of 0.5 mg/ml in 30 mM sodium carbonate and 68 mM sodium bicarbonate, pH 9.4. The plates were then blocked with 2% BSA in PBS. Serially diluted serum samples were added to the wells for 1 h at 37 °C to allow antibody binding. Goat anti-mouse IgG alkaline phosphatase conjugate (Sigma) was used to indicate bound antibodies. Enzymatic reactions were developed with *p*-nitrophenyl phosphate substrate. An SFV E1-specific mAb was used as a positive control. The assay cut-off value was calculated by adding three times the SD of the mean OD values taken from the sera of naive mice.

■ **Statistical analysis.** Analysis of variance was performed to compare the percentage of specific lysis or the CTL precursor frequency detected using spleen cells from the different vaccinated groups.

## Results

### *In vitro* studies

The NS3 gene was cloned into expression plasmids under the control of either the CMV promoter (pCI.NS3) or the SFV replicase machinery (rSFV.NS3). NS3 protein expression from pCI.NS3 and rSFV.NS3 was assayed in BHK-21 cells by Western blotting after transient transfection or infection. As shown in Fig. 1 (A, lanes 2 and 4), a protein with the expected molecular mass (70 kDa) was observed in cell extracts obtained after either transfection or infection of cells (Fig. 1A, arrows indicate NS3). Additional bands were observed: in particular, a

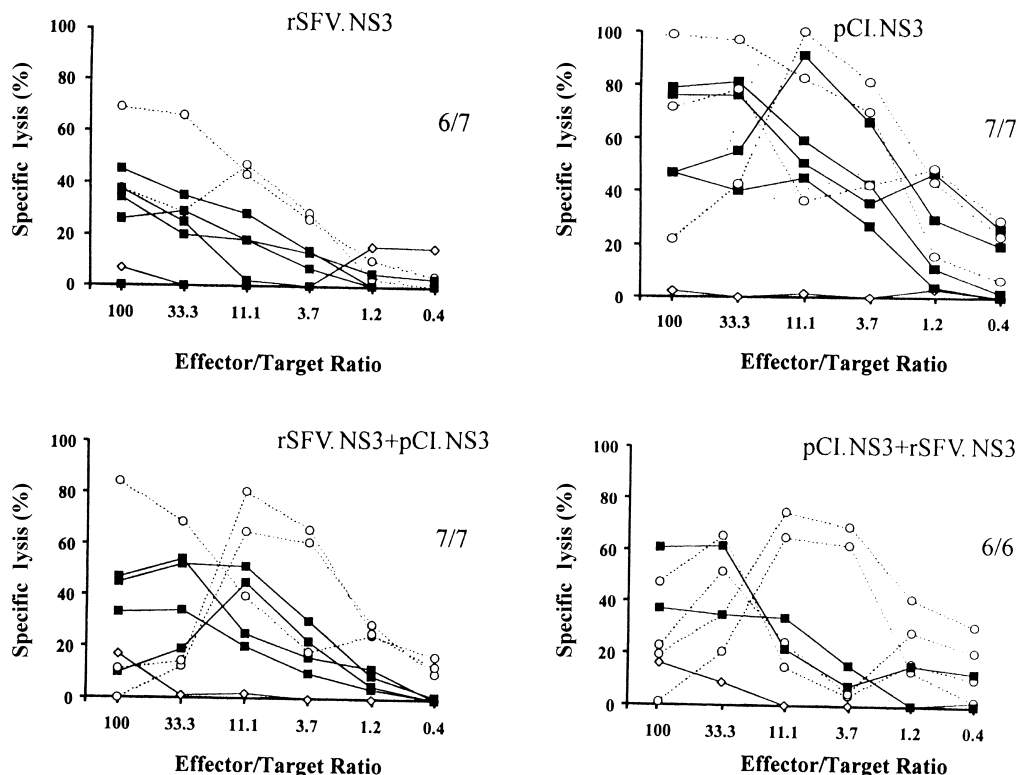


Fig. 3. CIN-specific CTL response following immunization with rSFV.NS3, pCI.NS3 or a combination of both. HLA-A2.1 transgenic mice received a total of four injections at 2 week intervals. pCI.NS3 and rSFV.NS3 mice were injected four times, while, in the case of combined vaccinations, mice received two injections of pCI.NS3 and two injections of rSFV.NS3 and vice versa. Bulk CTLs were analysed 4 weeks after the final injection, as described in Methods. Immunized mice from a first (■) and second (○) assay are represented. Mock-vaccinated mice (◇; rSFV.LacZ or pCI) are also indicated. The number of mice responding out of the number of mice tested is indicated in the upper right corner of each graph.

45 kDa protein that may correspond to the NS3a fragment was seen (Shoji *et al.*, 1999). Low molecular mass proteins were also evident, suggesting possible degradation products.

In order to quantify NS3 expression from the two vectors, flow cytometry experiments were performed. Analyses were carried out 24 h post-transfection or -infection (Fig. 1B). As predicted, rSFV.NS3 particles were shown to infect BHK-21 cells extensively (up to 75% of cells) compared with DNA transfection (21% of cells). At 24 h post-transfection, the level of antigen expressed from the two vectors appears to be quite similar, as characterized by a mean fluorescence intensity (MFI) of 337 for pCI.NS3 and 362.8 for rSFV.NS3. Transfections and infections of NIH 3T3 cells were also performed. While proper expression of the NS3 protein was observed with both pCI.NS3 and rSFV.NS3, the percentage of cells infected by the particles was, however, quite low (30%), rendering it more difficult to perform comparative analyses (data not shown).

The expression of the SFV replicase was investigated by flow cytometry (Fig. 1C) and was shown to be expressed 24 h after infection (78% infection with an MFI of 88.5).

All together, these results indicate that, under the experimental conditions used, the level of transduction observed with rSFV.NS3 is superior – as expected – to that obtained

with pCI.NS3. Nonetheless, the expression level of the antigen appears to be quite comparable between the two vectors, at least soon after transduction.

### Vaccination studies

In a natural infection, four HLA-A2.1-restricted epitopes contained in the NS3 protein have been reported to induce CD8<sup>+</sup>-mediated T cell responses in chronically infected patients and, in some cases, in patients recovering from self-limited infections (CIN, aa 1073–1081; KLV, aa 1406–1415; LLC, aa 1169–1177 and TGA, aa 1287–1296) (Battagay *et al.*, 1995; Cerny *et al.*, 1995; Kurokohchi *et al.*, 1996). We have demonstrated previously that the anti-NS3 CD8<sup>+</sup>-mediated immune response generated after DNA vaccination in the HLA-A2.1 transgenic mice was targeted mainly at one epitope (CIN, aa 1073–1081), while peptide-based vaccinations yielded specific CTLs for three of four epitopes (CIN, KLV and LLC), confirming that the mouse CTL repertoire for these epitopes is complete (the one peptide that did not induce CTLs does not, in fact, contain an HLA-A2-binding motif) (Brinster *et al.*, 2001). In the present study, rSFV.NS3 particles were also tested for their reactivity against the cited epitopes.

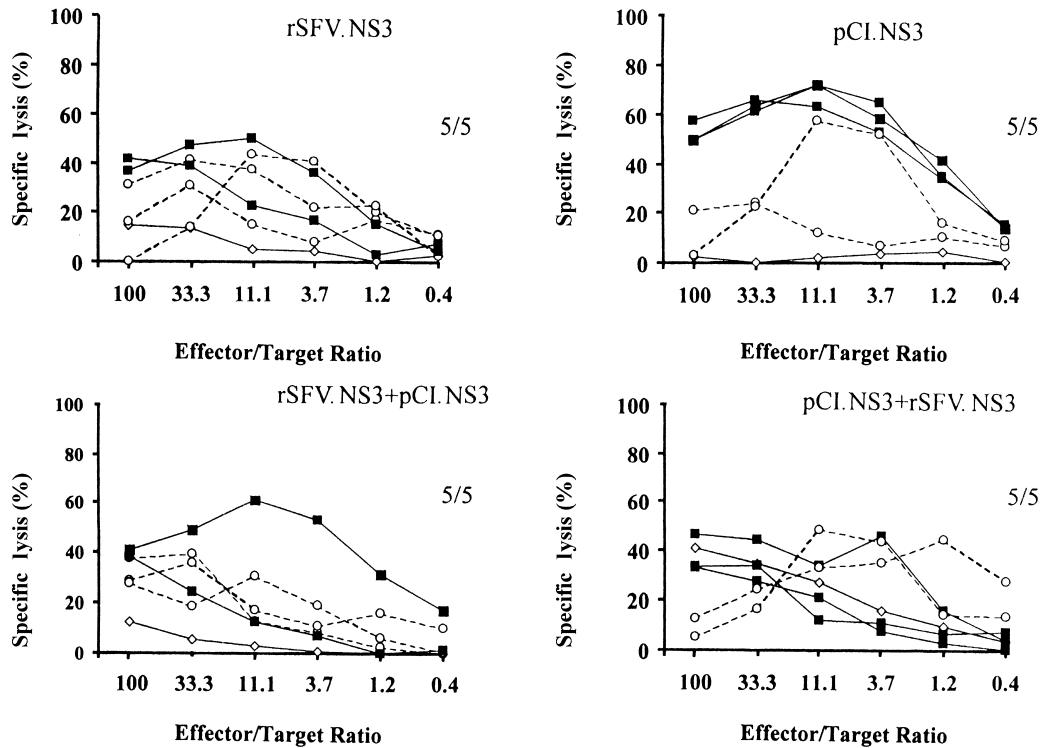


Fig. 4. Long-lasting induction of CIN-specific CTLs following vaccination with pCI.NS3, rSFV.NS3 or combined immunizations at 7 or 22 weeks after the final injection. HLA-A2.1 transgenic mice were immunized four times at 3 week intervals, as described in Fig. 3. Spleen cells were collected either at 7 (■) or 22 (○) weeks after the final injection. Bulk CTL analysis was performed as detailed in Methods. Mock-vaccinated mice (◇; rSFV.LacZ and pCI) are also indicated. The number of mice responding out of the number of mice tested is indicated in the upper right corner of each graph.

As described previously for pCI.NS3 (Brinster *et al.*, 2001), the CIN epitope and, to a more restricted extent, the LLC epitope, were targeted by the rSFV.NS3-induced CTLs obtained from vaccinated mice (Fig. 2).

We decided next to compare the CTLs induced against the CIN epitope following immunizations with rSFV.NS3, pCI.NS3 or a combination of both. In the first protocol, mice were injected four times at 2 week intervals with pCI.NS3 or rSFV.NS3. Combined immunizations were carried out by vaccinating mice two times with pCI.NS3 at 2 week intervals, followed by two injections of rSFV.NS3 at 2 week intervals and vice versa. Cytotoxic activity was assayed 4 weeks after the final injection. The results are represented in Fig. 3. Overall, all vaccine combinations were able to induce a detectable CTL response. pCI.NS3 was shown to induce a slightly more vigorous bulk CTL response, with 40–95% specific lysis at an E:T ratio of 33:3:1, compared to that induced by rSFV.NS3 ( $P = 0.0002$ ). rSFV.NS3 induced a lower response rate, with 0–70% specific lysis at an E:T ratio of 33:3:1 in six of seven mice responding. No significant difference in the induction of specific CTLs was observed in the combined schedules, with 20–70% specific lysis at an E:T ratio of 33:3:1, when compared with individual schedules ( $P > 0.5$ ).

In order to determine the persistence of the CTL response, a longer-term protocol was performed in which transgenic

mice were injected four times at 3 week intervals. CTL activity was performed at either 7 or 22 weeks after the final immunization (Fig. 4). As observed above, the rSFV.NS3 particles induced a somewhat weaker CIN-specific CTL activity than pCI.NS3, with 10–50% specific lysis compared with 20–70% at an E:T ratio of 33:3:1, respectively. The CTL activity observed after combined vaccinations displayed a vigour quite similar to that observed in the single vaccine regimens (20–45% specific lysis at an E:T ratio of 33:3:1). They remained detectable until 22 weeks after the final injection. The response rate of the vaccinated mice was optimal in each group (100%). None of the differences observed between the groups was statistically significant ( $P > 0.5$ ).

Overall, the CTL response developed against the CIN epitope was found to be quite vigorous at 7 weeks for all vaccine regimens tested and persisted up to 22 weeks after the final injection. No particular regimen appeared to be clearly superior, at least at the level of bulk CTL analysis.

#### Determination of the CIN-specific CTL precursor frequency following single or combined immunizations

A limiting dilution assay was carried out in order to characterize quantitatively the CIN-specific CTL response induced after the single or combined immunizations described

**Table 1.** CIN-specific CTL precursor frequency determined after immunizations with pCI.NS3, rSFV.NS3 or combinations of both

Mice were injected four times at 2 week intervals. The CIN-specific CTL precursor frequencies were determined using a limiting dilution assay (see Methods) 4 weeks after the final injection. Two independent and identical protocols (1 and 2) were performed.

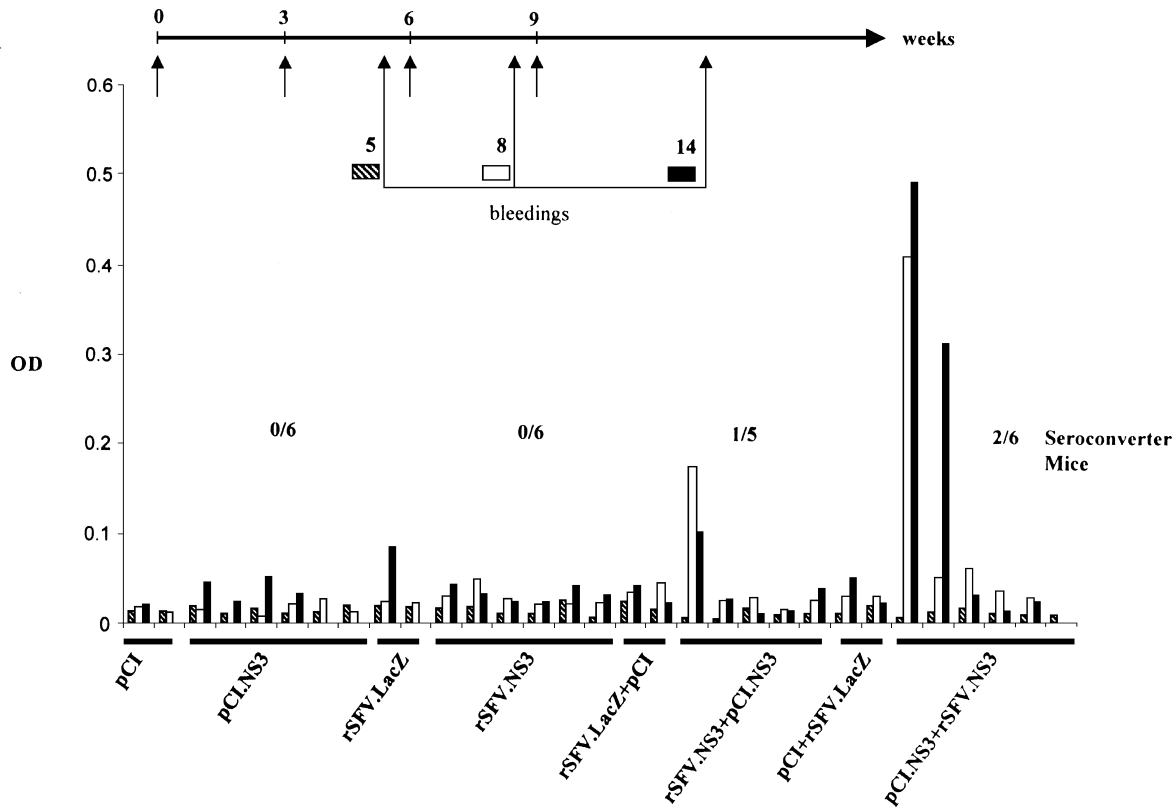
Immunogen	Protocol	CTL precursor frequency (1:total cells)	Mean value per protocol
rSFV.NS3	1	$\left\{ \begin{array}{l} 1:541729 \\ 1:1086941 \\ 1:509820 \\ 1:1304468 \end{array} \right.$	$1.8 \cdot 6 \times 10^5$
	2	1:804589	$1.8 \times 10^5$
rSFV.NS3 + pCI.NS3	1	$\left\{ \begin{array}{l} 1:804589 \\ 1:331574 \\ 1:638974 \\ 1:873032 \end{array} \right.$	$1.6 \cdot 6 \times 10^5$
	2	$\left\{ \begin{array}{l} 1:200375 \\ 1:322291 \\ 1:277563 \end{array} \right.$	$1.2 \cdot 6 \times 10^5$
pCI.NS3 + rSFV.NS3	1	ND	$1.1 \times 10^6$
	2	$\left\{ \begin{array}{l} 1:454779 \\ 1:2029799 \\ 1:2016014 \\ 1:106710 \end{array} \right.$	$1.1 \times 10^6$
pCI.NS3	1	$\left\{ \begin{array}{l} 1:592697 \\ 1:372710 \\ 1:381962 \end{array} \right.$	$1.4 \cdot 5 \times 10^5$
	2	$\left\{ \begin{array}{l} 1:221051 \\ 1:185430 \\ 1:130559 \end{array} \right.$	$1.1 \cdot 8 \times 10^5$

ND, Not determined as no mice responded.

above. Transgenic mice were injected four times at 2 week intervals and CTLs were analysed 4 weeks after the final immunization. For each combination of vector, two identical but independent protocols were performed (Table 1, protocols 1 and 2). In the first protocol, no CIN-specific CTL precursor frequency could be obtained for the pCI.NS3 + rSFV.NS3 combination, as no mice responded to the vaccination at that time of analysis. Among the three other groups tested, mice immunized with pCI.NS3 were shown to induce the higher CTL precursor frequency (mean of  $1.4 \cdot 5 \times 10^5$ , with values ranging from 1:381962 to 1:592697) compared with rSFV.NS3 (mean of  $1.8 \cdot 6 \times 10^5$ , with values ranging from 1:509820 to 1:1304468;  $P = 0.06$ ) and rSFV.NS3 + pCI.NS3 (mean of  $1.6 \cdot 6 \times 10^5$ , with values ranging from 1:331574 to 1:873032;

$P = 0.10$ ). In the second protocol, similar levels of response were found as compared to those obtained in the first protocol. Mean precursor frequencies varied from  $1.1 \times 10^6$  for pCI.NS3 + rSFV.NS3-based injections to  $1.8 \times 10^5$ – $1.8 \cdot 6 \times 10^5$  for rSFV.NS3-based injections. Overall, while some discrepancies were observed between the different vaccinated groups, they remained below 1 log.

Using ELISpot-based assays, we were unable to detect any interferon (IFN)- $\gamma$ -producing T cells in mice immunized either with rSFV.NS3 particles or with the different vaccine combinations (data not shown). This lack of IFN- $\gamma$  production was observed whatever the HLA-A2.1 epitope (CIN, KLV, TGA or LLC) tested and whether the assay was run *ex vivo* or after one round of *in vitro* stimulation with the different peptides (data



### Immunogens and vaccine regimens

Fig. 5. Anti-NS3-specific antibodies in immunized BALB/c mice. BALB/c mice were immunized four times at 3 week intervals with the different vectors (pCI.NS3 or rSFV.NS3) or combinations of both. Sera were collected at 5, 8 and 14 weeks after the initial injection and analysed as described in Methods. Results are given as OD values.

not shown). In contrast, we reproducibly detected IFN- $\gamma$ -producing cells in these mice following vaccination with core- or E2-expressing plasmids or following vaccination with peptides representing HLA-A2 NS3-specific epitopes (N. Himoudi, personal communication; Brinster *et al.*, 2001).

### Humoral immune responses

No anti-NS3-specific antibodies could be detected in the vaccinated transgenic mice, irrespective of the vector used or the protocol implemented. Nonetheless, when another haplotype of BALB/c mice ( $H-2^d$ ) was tested, anti-NS3 antibodies could be detected in the context of combined immunizations. This response was, nonetheless, low (one of five and two of six responder mice; Fig. 5). In contrast to these observations, in both haplotypes of mice, vaccination with a soluble recombinant NS3 protein induced detectable antibodies with a 100% seroconversion rate (data not shown; Brinster *et al.*, 2001).

### Anti-SFV humoral immune response

Among the combined vaccinations tested, only the rSFV.NS3 particles were shown to induce antibodies specific for

the SFV particles at 8 and 14 weeks after the final injection (i.e. following the third and fourth injection) (Table 2).

### Discussion

Since SFV replication-defective recombinant particles (rSFV) have been reported previously to induce better immune responses than DNA/RNA SFV-derived vaccines in virus models, such as HIV or LIV (Brand *et al.*, 1998; Fleeton *et al.*, 2000), we were interested in testing the potential of rSFV particles expressing NS3 (rSFV.NS3) for the induction of specific immune responses. For this purpose, rSFV.NS3 particles and a DNA-based vaccine (pCI.NS3) were compared in HLA-A2.1 transgenic mice in single or combined immunizations.

The first observation that we made was that rSFV.NS3 particles can induce a specific, although narrow, CD8<sup>+</sup> CTL-mediated response in our transgenic mice, which have the unique characteristic of being totally devoid of murine MHC class I molecules (Pascolo *et al.*, 1997). Indeed, a predominant HLA-A2.1-restricted epitope known to exist in HCV-infected patients was the target of CTLs after vaccination of the mice (CIN, aa 1073–1081). We also observed a similar restriction

**Table 2.** Titration of IgGs specific for rSFV particles following immunization with rSFV.NS3 or combination regimens

Transgenic mice were immunized four times at 3 week intervals. Sera were collected at 8 and 14 weeks after the first immunization. Antibody titres were considered positive when data were above the value 1:100. Each immunization group contained three to five mice.

Immunogen	Time after initial injection (weeks)	Anti-SFV titre	Reactivity
rSFV.LacZ	8	< 1:100	–
	14	1:2500	+
rSFV.NS3	8	1:2500	+
		1:2500	+
		1:100	+
		< 1:100	–
	14	< 1:100	–
		1:100	+
		1:2500	+
		1:2500	+
pCI + rSFV.LacZ	8	< 1:100	–
	14	1:2500	+
pCI.NS3 + rSFV.NS3	8	< 1:100	–
		< 1:100	–
		< 1:100	–
		< 1:100	–
	14	< 1:100	–
		< 1:100	–
		< 1:100	–
		< 1:100	–
rSFV.LacZ + pCI	8	< 1:100	–
	14	< 1:100	–
rSFV.NS3 + pCI.NS3	8	< 1:100	–
		< 1:100	–
		< 1:100	–
		< 1:100	–
	14	< 1:100	–
		< 1:100	–
		< 1:100	–
		< 1:100	–

pattern in mice vaccinated with the NS3 DNA-based vaccine (Brinster *et al.*, 2001). It is difficult to attribute this restriction to a problem with antigen processing and presentation in the mouse model used. Indeed, recent studies, one performed by Loirat *et al.* (2000) with the HBV model and one performed by Woodberry *et al.* (1999) with the HIV model, have shown a very good correlation between the vigour and the number of CTL epitopes induced in mice following DNA vaccination and those seen in HBV- or HIV-infected patients. In our study, vaccination using rSFV or DNA seems to be quite similar overall in terms of vigour and breadth of induced specific immune responses, possibly because they involve similar

mechanisms for antigen stimulation. We chose the intravenous route to inject rSFV particles, as it was found to be the most efficient route of inoculation in the influenza virus model (Berglund *et al.*, 1999). We also used the intraperitoneal route in later experiments, but no differences could be observed in terms of the number of reactive mice and vigour of the CTLs induced between the two routes (data not shown). Comparison with additional vaccine vehicles is warranted to try and broaden an NS3-specific CD8<sup>+</sup>-mediated immune response such that multiple (HLA-A2) epitopes are targeted simultaneously. Of consideration is the fact that our NS3 vaccines are limited to the NS3 sequence only and do not contain other

flanking HCV sequences that may modify its processing, as might take place in a natural infection and/or may contain important helper epitopes that would be missing in our NS3 vaccines.

Our observations using rSFV particles contrast with some reports described in other virus models for which immune responses induced by such particles were generally better than those observed with DNA-based vaccines. Notably, this was the case for LIV, where rSFV particles expressing the envelope prME or NS1 antigens were shown to efficiently protect mice compared with SFV-derived or CMV-driven DNA plasmids (Fleeton *et al.*, 2000). In the HIV model, rSFV particles expressing the HIV-1 envelope protein were also shown to induce a stronger humoral response than a recombinant envelope glycoprotein, a DNA vaccine or rSFV RNA (Brand *et al.*, 1998). Recombinant alphavirus particles are also efficient at inducing significant protection in mice or primates with other models, such as influenza virus (Berglund *et al.*, 1999; Zhou *et al.*, 1995), LIV (Fleeton *et al.*, 1999), HIV (Berglund *et al.*, 1997), simian immunodeficiency virus (SIV) (Davis *et al.*, 2000; Mossman *et al.*, 1996), HPV (Daemen *et al.*, 2000), Japanese encephalitis virus (Pugachev *et al.*, 1995), malaria infection (Tsuji *et al.*, 1998) or cancer studies (Colmenero *et al.*, 1999). Nonetheless, two cases, Murray Valley encephalitis virus (Colombage *et al.*, 1998) and hantaviruses (Kamrud *et al.*, 1999), have also been reported where recombinant alphavirus particles failed to protect animals from a virus challenge, while DNA-based vaccines had a protective effect. These apparent discrepancies are likely to be the results of specific interferences between the SFV expression system itself and the nature of the immunogens expressed. For example, it is known that cells infected by rSFV are subject to apoptosis (Glasgow *et al.*, 1997). As NS3 has been shown to possess anti-apoptotic properties (Ishido & Hotta, 1998; Sakamuro *et al.*, 1995), interference at that level could affect the persistence of antigen expression characteristic of the SFV system. A recent study examining the persistence and distribution of a reporter gene (green fluorescent protein) following immunizations with rSFV particles or DNA-based vaccines in mice and chickens has demonstrated that the antigen expression elicited from rSFV was very transient and localized to the injection site, while that observed after DNA vaccine was persistent (up to months) and distal from the injection site (Morris-Downes *et al.*, 2001). Overall, caution should be taken when trying to link *in vitro* observations with *in vivo* observations, as a higher level of *in vitro* expression may not be representative of the situation *in vivo*.

Another explanation for the somewhat limited immune response observed after vaccinations performed with rSFV.NS3 alone could be due to the anti-SFV immune response. We observed the development of anti-SFV antibodies in 80% of vaccinated mice (Table 2), which could be responsible for the inhibition of a booster effect. Berglund *et al.* (1999) reported recently the lack of SFV-specific humoral responses using

influenza virus-derived rSFV particles, but Kamrud *et al.* (1999) and Mossman *et al.* (1996) both demonstrated in hantavirus or SIV models that such anti-SFV antibodies could lead to the reduction of the antigen-specific cellular immune response. In our study, the neutralizing effect of anti-SFV antibodies may, nonetheless, be limited, as three immunizations were necessary before antibodies could be detected.

We were unable to detect any CD8<sup>+</sup>-derived IFN- $\gamma$ -producing T cells following immunizations with pCI.NS3, rSFV.NS3 or the combined regimen using the ELISpot technique. It is possible that other cytokines might have been induced (e.g. a Th2 type) but these were not evaluated here. Our results are consistent with the observations made by Rehermann and co-workers using an NS3 DNA-based vaccine (B. Rehermann, personal communication), but are in contrast to those found using live attenuated *Salmonella typhimurium*. Such a vector, used in the form of DNA in HLA-A2.1 transgenic mice, was capable of inducing CD8<sup>+</sup> T cells (Wedemeyer *et al.*, 2000). The lack of detectable specific IFN- $\gamma$ -producing lymphocytes may be compared with the natural situation during chronicity: Prezzi *et al.* (2001) reported an incapacity to detect *ex vivo* HCV-specific liver-infiltrating lymphocytes using techniques such as ELISpot or tetramer staining in chronically infected patients. Such cells are likely to exist, but at a very low frequency. We have shown previously that vaccination of transgenic mice with NS3 HLA-A2 peptides results in the production of IFN- $\gamma$  detectable in *ex vivo* ELISpot assays (Brinster *et al.*, 2001). Thus, a peptide-based vaccine appears to induce a more vigorous cellular immune response than a DNA- or an rSFV-based one, a response possibly mimicking more closely that observed during resolved infection. Finally, a recent study by Pancholi *et al.* (2000) reports another DNA-prime/virus vector boosting vaccination regimen in the HCV model. This study reveals a profound influence of the line of mouse used on the NS3-specific cellular immune responses detected. While vaccination of mice (BALB/c or C57BL/6) with a recombinant NS3-expressing canarypox virus failed to induce detectable antibodies or specific cellular immune responses, a DNA/canarypox virus combined vaccine regimen showed a significant and enhanced induction of IFN- $\gamma$ -producing cells in BALB/c mice only. Our study remains limited in that we used only one line of transgenic mice, which happens to be C57BL/6.

In conclusion, NS3-expressing rSFV particles induce rather similar results in terms of vigour and breadth of specific cellular immune responses as those observed with an NS3 DNA-based vaccine. This study is the first report on the use of rSFV particles in a transgenic mouse model. While our results do not show any enhancement in the induction of anti-NS3-specific immune responses when rSFV particles are used compared with naked DNA, they illustrate the capacity of the particles to induce NS3-specific CTLs capable of recognizing what appears to be a dominant HLA-A2 epitope in a natural infection. Other vaccine strategies based on the combination of rSFV with other

types of NS3-expressing virus vectors (such as adenovirus, modified vaccinia virus Ankara-derived or recombinant proteins) or including additional HCV sequences may need to be developed in order to obtain a broader and enhanced anti-NS3 immune response.

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