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ORIGINAL ARTICLE

Long-term functional duration of immune responses to HCV NS3/4A induced by DNA vaccination

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We have investigated the ability of hepatitis C virus non-structural (NS) 3/4A-DNA-based vaccines to activate long-term cell-mediated immune responses in mice. Wild-type and synthetic codon optimized (co) NS3/4A DNA vaccines have previously been shown to be immunogenic in mice, rabbits and humans, although we have very poor knowledge about the longevity of the immune responses primed. We therefore analyzed the functionality of primed NS3/4A-specific immune responses in BALB/c (H-2^d) and/or C57BL/6J (H-2^b) mice 1, 2, 3, 4, 6, 12 and 16 months after the last immunization. Mice were immunized one, two, three or four times using gene gun delivery to the skin or by intramuscular administration. Immunological responses after immunization were monitored by protection against *in vivo* challenge of NS3/4A-expressing syngeneic tumor cells. In addition, functionality of the NS3/4A-specific T cells was analyzed by a standard cytotoxicity assay. First, we identified a new unique murine H-2^d-restricted NS3/4A cytotoxic T lymphocyte (CTL) epitope, which enabled us to study the epitope-specific immune responses. Our results show that the coNS3/4A vaccine was highly immunogenic by determination of interferon-γ/tumor necrosis factor-α production and lytic cytotoxic T cells, which could efficiently inhibit *in vivo* tumor growth. Importantly, we showed that one to four monthly immunizations protected mice from tumor development when challenged up to 16 months after the last immunization. When determining the functionality of NS3/4A-specific T cells *in vitro*, we showed detectable lytic activity up to 12 months after the last immunization. Thus, NS3/4A-based DNA vaccines activate potent cellular immune responses that are present and function in both BALB/c and C57BL/6J mice up to 12–16 months after the last immunization. The induction of long-term immunity after NS3/4A DNA immunization has not been shown previously and supports the use of NS3/4A in hepatitis C virus vaccine compositions.

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INTRODUCTION

Hepatitis C virus (HCV) infection is one of the leading causes of chronic liver disease worldwide. It has been estimated that around 185 million individuals globally have anti-HCV antibodies and of these 130–170 million are chronic carriers of the infection.[1,2] The HCV belongs to the *Flaviviridae* virus family and is grouped into the genus *Hepacivirus*.[3] The HCV exist in seven major genotypes (1–7) and numerous subtypes (a, b, c, etc.).[3] It has a single-stranded positive-sense RNA molecule, which encodes a single large polyprotein. This polyprotein undergoes proteolytic cleavage by cellular and viral proteases, which process the polyprotein into 10 structural- and non-structural proteins. The current treatment for HCV is based on pegylated interferon-α (peg-IFNα) and ribavirin with the addition of direct acting anti-virals (DAAs, e.g. protease and polymerase inhibitors).[4–8] Numerous DAAs are in clinical development and will be approved during the coming years.[9,10] The addition of DAAs to the current peg-IFNα and ribavirin treatment has shown improved sustained virologic responses in genotype 1 patients.[6] However, the addition of new DAAs to the HCV treatment has also increased the cost of treatment. Hence, resource-poor countries may not be able to afford the new improved treatments. In addition, it has been estimated that only one-tenth of all HCV-positive individuals receive treatment.[2] Thus, alternative treatment strategies are needed both for non-responder patients and also as a treatment option in resource-poor countries. One treatment option that has been explored is therapeutic vaccination. However, no vaccine is currently available, although several vaccines have been tested in clinical trails.[11–15] We have developed genotype 1 HCV non-structural 3/4A (NS3/4A)-based genetic vaccines, which have been evaluated in mice, rabbits and humans.[15–17] The NS3/4A protein complex of HCV has important protease and helicase activities and participates in the replication module together with NS4B, NS5A and NS5B. The NS3/4A region of HCV was chosen as a vaccine candidate because previous studies have shown that NS3-specific T-cell responses correlate with resolution of infection.[18–20] Our data showed that NS3/4A-based vaccines were safe, immunogenic and had transient effects on viral load.[15–17] Genetic vaccines are known to induce both humoral and cellular immune responses in animals, including humans. Patients who spontaneously or by treatment clear HCV infection commonly have $CD4^+$ and $CD8^+$ T-cell responses to multiple HCV proteins, whereas these types of immune responses are weak or absent in patients with a chronic HCV infection. Thus, the therapeutic vaccination should activate naive T cells or reactivate the dysfunctional T cells

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present in the infected individuals and thereby control and/or clear the HCV infection.[21]

In this study, we were particularly interested in analyzing the longevity of HCV NS3/4A-specific immune responses after immunization. The herein used NS3/4A vaccine has previously been evaluated in a phase I/II clinical trial.[15] We also extended the monitoring of immune responses to two different mouse haplotypes and strains, different administration routes and antigen doses to enhance the understanding of immune activation by genetic vaccines.

RESULTS

Identification of an H-2^d-restricted epitope in HCV NS3/4A

We have previously identified an NS3 major histocompatibility complex (MHC) class I CTL epitope in C57BL/6J (H-2^b) mice that bound H-2D^b molecules with high affinity,[22] which has been used for monitoring NS3/4A-specific CTL responses. In the present study, we wanted to expand the immunological analysis of NS3/4A-specific immune responses by evaluating immune responses to NS3/4A in another mouse haplotype. To evaluate NS3/4A-based DNA vaccines in BALB/c mice, we needed to identify an H-2^d-restricted NS3/4A CTL epitope. To identify new MHC class I epitopes within the NS3/4A protein, we synthesized 68 20-mer peptides (10-amino-acid (aa) overlap) spanning the entire NS3/4A protein. All 68 peptides were screened for binding to the H-2^d molecule as determined by stabilization of MHC–peptide complexes on transporter associated with antigen processing 2-deficient RMA-S cells[23,24] transfected with H-2K^d, H-2D^d or H-2L^d. This extensive analysis revealed one 20-mer peptide (e.g. amino-acid sequence: TVRLRAYMNTPGLPVCQDHL) that could stabilize the H-2K^d molecule. This same region has been suggested to contain a CTL epitope previously, but it has not been fine mapped.[25] Hence, we decided to fine map this NS3/4 A-specific H-2^d CTL epitope. To fine map the H-2^d epitope, a panel of ten 10-mer peptides with 9 aa overlap was synthesized (Supplementary Figure 1A). To determine IFN-γ production in response to the 10-mer peptides, BALB/c mice were immunized once with 100 μg coNS3/4A-pVAX1 intramuscularly, and 2 weeks later the animals were killed. Spleen cells were restimulated for 4 h *in vitro* in the presence of the 10-mer peptides and the frequency of IFN-γ-producing CD8+ T cells was determined by flow cytometry (Supplementary Figure 1B). We could identify two peptides (numbers 3 and 5) that were able to induce high IFN-γ production by CD8+ T cells (Supplementary Figure 1B). At the same time, we performed an *in vitro* peptide stabilization of MHC–peptide complexes on transporter associated with antigen processing 2-deficient RMA-S cells transfected with H-2K^d, H-2D^d or H-2L^d. Our results revealed that only peptides 3, 4, 5 and 6 could bind to the H-2K^d molecule and not the other two H-2 alleles (Supplementary Figure 1B). Moreover, we analyzed the cytotoxic potential of spleen cells from BALB/c coNS3/4A-pVAX1 or peptide-immunized mice by performing a 5-day restimulation using peptides 3, 4 and 5. The cytolytic activity of the NS3/4A-specific CTLs was determined by a conventional ^{51}Cr-release assay on peptide-loaded RMA-S target cells. Functional testing revealed that all three peptides bound to MHC class I and were recognized and eradicated by NS3/4A-specific CTLs (data not shown). Based on all the above data, peptide number 5 (amino-acid sequence: AYMNTPGLPV, positions 1541–1550 in the HCV polyprotein) was selected for all further experimental procedures as it had the highest binding affinity for the H-2K^d molecule and it also induced high levels of IFN-γ production by CD8+ T cells. To investigate the specificity of the identified CTL epitiope, we immunized groups of five BALB/c (H-2^d), C57BL/6J × BALB/c (H-2^d × H-2^b) and C57BL/6J (H-2^b) mice two times with 2 μg coNS3/4A-pVAX1 using transdermal delivery via gene gun. Groups of mice were also left untreated as controls. Two weeks after the last immunization, mice were killed and splenocytes were restimulated with either the H-2^d peptide (AYMNTPGLPV) or the previously described H-2^b peptide (GAVQNEVTL).[22] Restimulated cultures were analyzed for lytic activity by a ^{51}Cr-release assay using peptide-loaded RMA-S cells (Figures 1a–h). The results revealed that NS3/4A-specific CTLs with lytic activity were only detected in BALB/c and C57BL/6J × BALB/c cultures restimulated with the H-2^d peptide (Figures 1a and c), whereas no lytic activity were detected in C57BL/6J cultures (Figure 1e). On the other hand, when splenocytes from immunized C57BL/6J mice were restimulated with the H-2^b peptide, similar lytic activity was noted (Figure 1g). Hence, the H-2^d-binding peptide is only presented in the context of BALB/c mice. No lytic activity was detected in the groups of non-immunized mice (Figures 1b, d, f and h).

NS3/4A immunization primes tumor-inhibiting immune responses

Next, we wanted to evaluate the efficiency of the *in vivo* primed NS3/4A-specific CTL responses. We therefore used a tumor inhibition model, in which we can characterize the *in vivo* functionality of the NS3/4A-specific cellular immune response by determining the inhibition of tumor growth *in vivo*. We have previously established two cell lines stably expressing NS3/4A,[17,22] which enable us to evaluate tumor-inhibiting immune responses after NS3/4A immunization. Protection against NS3/4A-expressing EL-4 lymphoma cells were evaluated in C57BL/6J mice and protection against NS3/4A-expressing Sp2/0 myeloma cells were evaluated in BALB/c mice. First, we were interested in the *in vivo* growth characteristics of these cell lines in naive C57BL/6J mice and BALB/c mice. To determine if the stably transfected cell lines have similar growth properties compared with the parental cell lines, we inoculated equal numbers of cells into groups of 5–7 C57BL/6J or BALB/c mice. We noted that the four cell lines had similar *in vivo* growth curves with palpable tumors from around day 5 after inoculation. Without any treatment, the tumors continued to grow for more than 2 weeks after inoculation. No difference in growth properties between the NS3/4A-Sp2/0 and the parental Sp2/0 cell lines were seen (P = NS; Supplementary Figure 2A). Moreover, we did not see any differences between the growth properties of the NS3/4A-EL-4 and the parental EL-4 cell lines (P = NS; Supplementary Figure 2B). Next, we were interested in whether DNA immunization using NS3/4A-based vaccines could protect mice from tumor growth. Specifically, we immunized groups of 8–10 C57BL/6J mice once by gene gun with 2 μg of wild-type (wt)NS3-pVAX1, wtNS3/4A-pVAX1 or coNS3/4A-pVAX1 DNA vaccines. Control mice were left non-immunized. Two weeks after immunization, mice were challenged with a subcutaneous inoculation of 1×10^6 NS3/4A-EL-4 cells, and tumor growth was monitored for up to 3 weeks (Figure 2a). Our results show that NS3/4A-based vaccination mediated protection against tumor growth ($P < 0.001$), whereas NS3-based vaccines protected less efficiently against tumor growth, although statistically significantly ($P < 0.05$; Figure 2a; Frelin *et al*.[17,22]). Thus, inclusion of the NS3 cofactor NS4A is essential for optimal NS3 immunogenicity. Similarly, groups of 9–10 BALB/c mice were immunized two or four times at monthly intervals with 2 μg of wtNS3/4A-pVAX1 or coNS3/4A-pVAX1 using gene gun. Two weeks after the last immunization, mice were challenged with a subcutaneous inoculation of 1×10^6 NS3/4A-Sp2/0 cells and thereafter tumor growth was monitored for up to 3 weeks. All groups of immunized mice were protected against tumor growth, whereas non-immunized mice developed tumors ($P < 0.01$ and < 0.001). There was no significant differences between mice immunized with wtNS3/4A-DNA or coNS3/4A-DNA (Figure 2b). Moreover, no statistical difference was found between groups of mice immunized two or four times (Figure 2b). In addition to monitoring the *in vivo* functionality of the vaccine-primed T-cell responses, we were also interested in determining the *in vitro* lytic

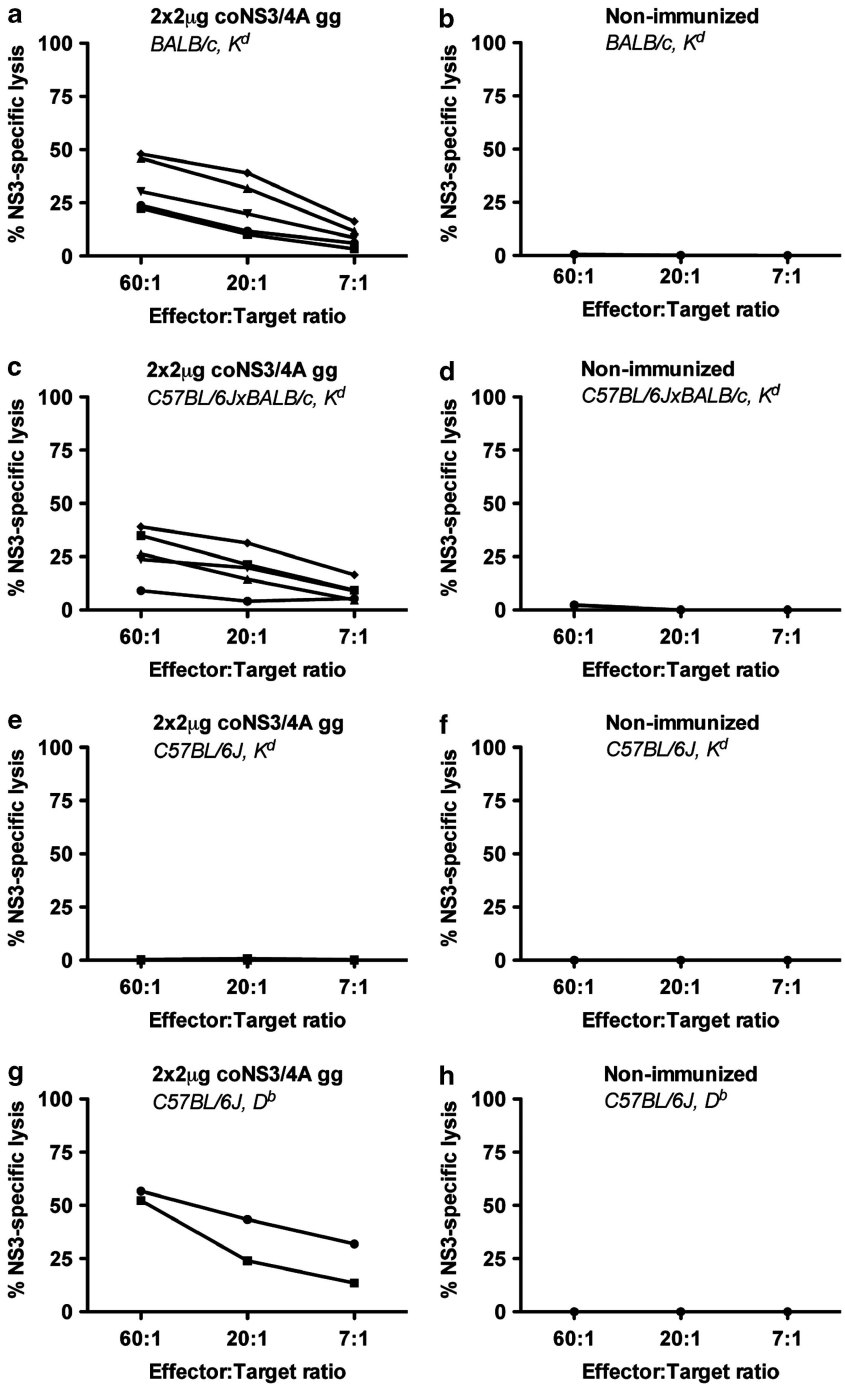
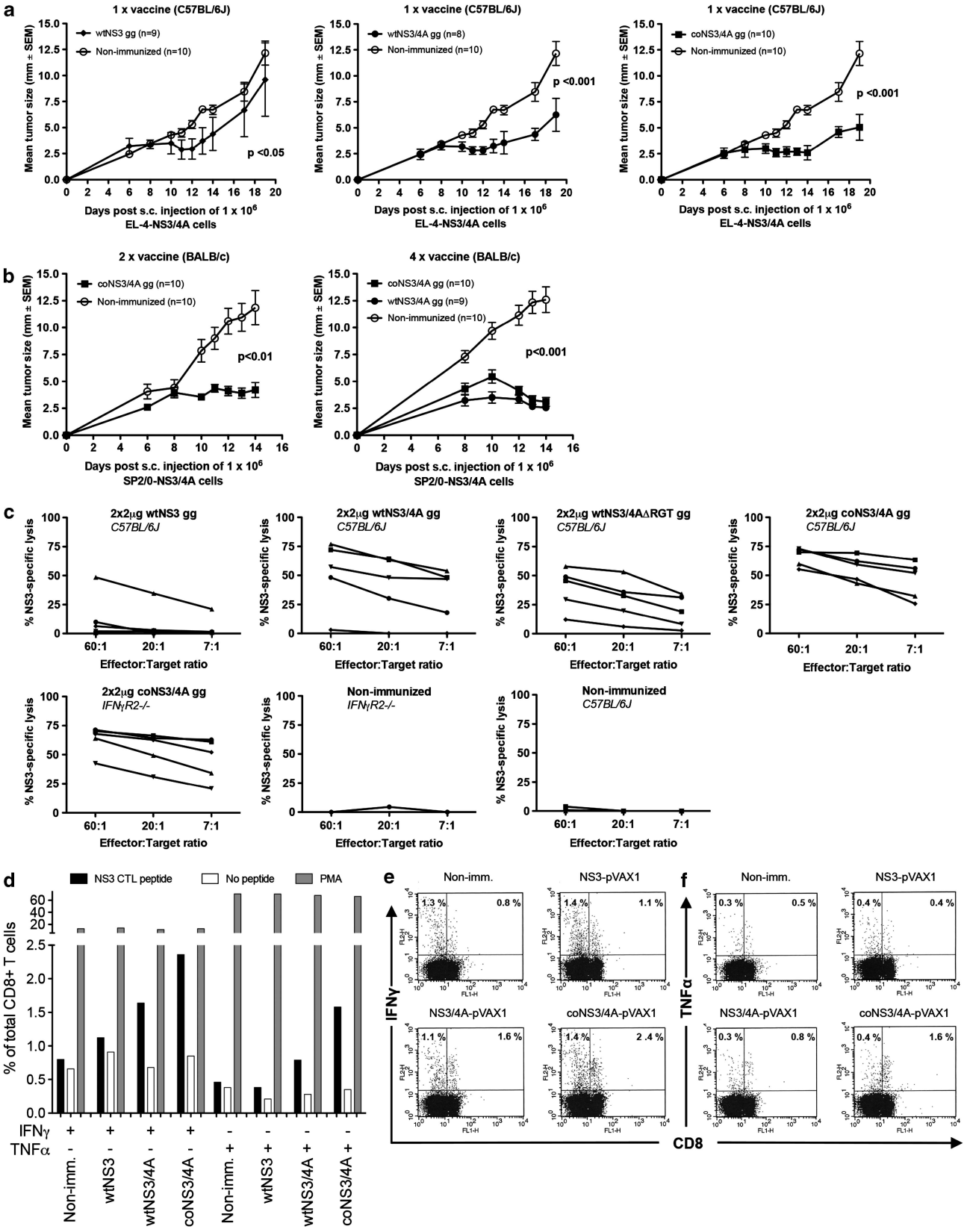


Figure 1. Priming of *in vitro* detectable CTLs against K^d- and D^b-binding peptides in H-2^d (BALB/c) and H-2^b (C57BL/6J) mice. Groups of five mice were immunized two times with 2 μg coNS3/4A plasmid DNA using transdermal gene gun (gg) delivery (**a**, **c**, **e** and **g**) or left untreated (non-immunized) (**b**, **d**, **f** and **h**). Two weeks after the last immunization, the specific lytic activity was determined using peptide-loaded RMA-S cells at E:T ratios of 60:1, 20:1 and 7:1 in a standard ^{51}Cr-release assay. Specific lysis above 10% was considered positive. Each line indicates an individual mouse.

activity of NS3/4A-based DNA vaccines in wt and IFNγR2 − / − mice. We therefore immunized groups of five mice two times with 2 μg of wtNS3-pVAX1, wtNS3/4A-pVAX1 or wtNS3/4AΔRGT-pVAX1 (construct with deficient NS3 proteolytic activity), or coNS3/4A-pVAX1. Again, only NS3 constructs including NS4A primed potent NS3-specific lytic activity. There was no significant difference in the *in vitro* lytic activity between plasmids with a functional or a deficient NS3 protease (Figure 2c), although the trend was toward less lytic activity with a deficient protease. Moreover, C57BL/6J and IFNγR2 − / − mice immunized with coNS3/4A-pVAX1 primed equally strong lytic activities. Mice were also immunized three times with similar results (data not shown). We were also interested in the cytokine profile of mice immunized with NS3/4A-based vaccines. We found that vaccines containing NS3 and NS4A were superior with regard to the amount of cytokines produced upon *in vitro* stimulation (Figures 2d–f). The coNS3/4A-pVAX1 vaccine produced the highest levels of IFN-γ and TNFα followed by wtNS3/4A-pVAX1. Mice immunized with

wtNS3-pVAX1 rarely had any production of cytokines with results similar to non-immunized controls. Immunized and non-immunized mice stimulated without peptide had similar levels of IFN-γ and TNFα-production as non-immunized mice stimulated with the NS3-CTL peptide. Representative dot plots of flow cytometry analysis are shown in Figures 2e and f.



A key question to address was how long after the last immunization could T cells remain functional in mice? To answer this question, we immunized BALB/c and/or C57BL/6J mice one to three times with wtNS3/4A-pVAX1 or coNS3/4A-pVAX1 DNA vaccines and thereafter waited 1, 2, 3, 4, 6, 12 and 16 months. At indicated time points, mice were challenged with NS3/4A-expressing tumor cells or splenocytes were monitored *in vitro* for detection of CTL responses (Figures 3a and b). We found that one immunization protected C57BL/6J mice from tumor development when challenged 1, 3 and 6 months after the last immunization ($P < 0.001$; Figure 3a). Not surprisingly, mice immunized three or four times were protected against tumor growth when challenged 4 months after the last immunization (data not shown). In addition, three monthly immunizations protected BALB/c mice from tumor development when challenged 16 months after the last immunization irrespective of administration route ($P < 0.01$; Figure 3a). Importantly, we obtained similar results regarding long-term protective immune responses in two mouse strains. When determining the functionality of NS3/4A-specific T cells *in vitro*, we showed detectable lytic activity up to 12 months after the last immunization in groups of C57BL/6J mice immunized two times (Figure 3b). Additionally, in groups of mice immunized once, we detected lytic activity for up to 6 months (Figure 3b). However, the lytic activity was at a lower magnitude after one immunization compared with two immunizations. Next, we were interested in comparing NS3/4A-specific immune activation in wt and IFNγR2−/− mice as determined by IFN-γ and IL-2 production by Enzyme-Linked ImmunoSpot (ELISpot), lytic activity by a cytotoxicity assay and quantification of NS3/4A-specific T cells using pentamer staining. Groups of five mice were immunized once with gene gun (2 μg) or intramuscular immunization (50 μg) and 2 weeks later were killed and immune responses subsequently monitored. We noted that wt mice immunized intramuscularly primed stronger IFN-γ production compared with mice immunized transdermally using gene gun (Figure 4a). As expected, no IFN-γ production was detected in IFNγR2−/− mice (Figure 4a). However, we found detectable levels of IL-2-production in both wt and IFNγR2−/− mice immunized intramuscularly, whereas the IL-2 production were weak or absent in mice immunized transdermally using gene gun (data not shown). To determine if the primed NS3/4A-specific T cells were functional, a cytotoxicity assay was used. Both immunized wt and IFNγR2−/− mice had detectable CTLs that were able to lyse NS3/4A target cells (Figure 4a). However, it was noted that IFNγR2−/− mice immunized transdermally had lower levels of CTLs with lytic activity. Quantification of NS3-specific CD8+ T cells revealed that wt mice immunized intramuscularly or transdermally, and IFNγR2−/− mice immunized intramuscularly primed similar frequencies of T cells, whereas IFNγR2−/− mice immunized transdermally had low frequencies of positive T cells ($P < 0.05$ (wt) and $P < 0.01$ (IFNγR2−/−); Figure 4b). Also, representative dot plots of flow cytometry analysis are shown (Figure 4b).

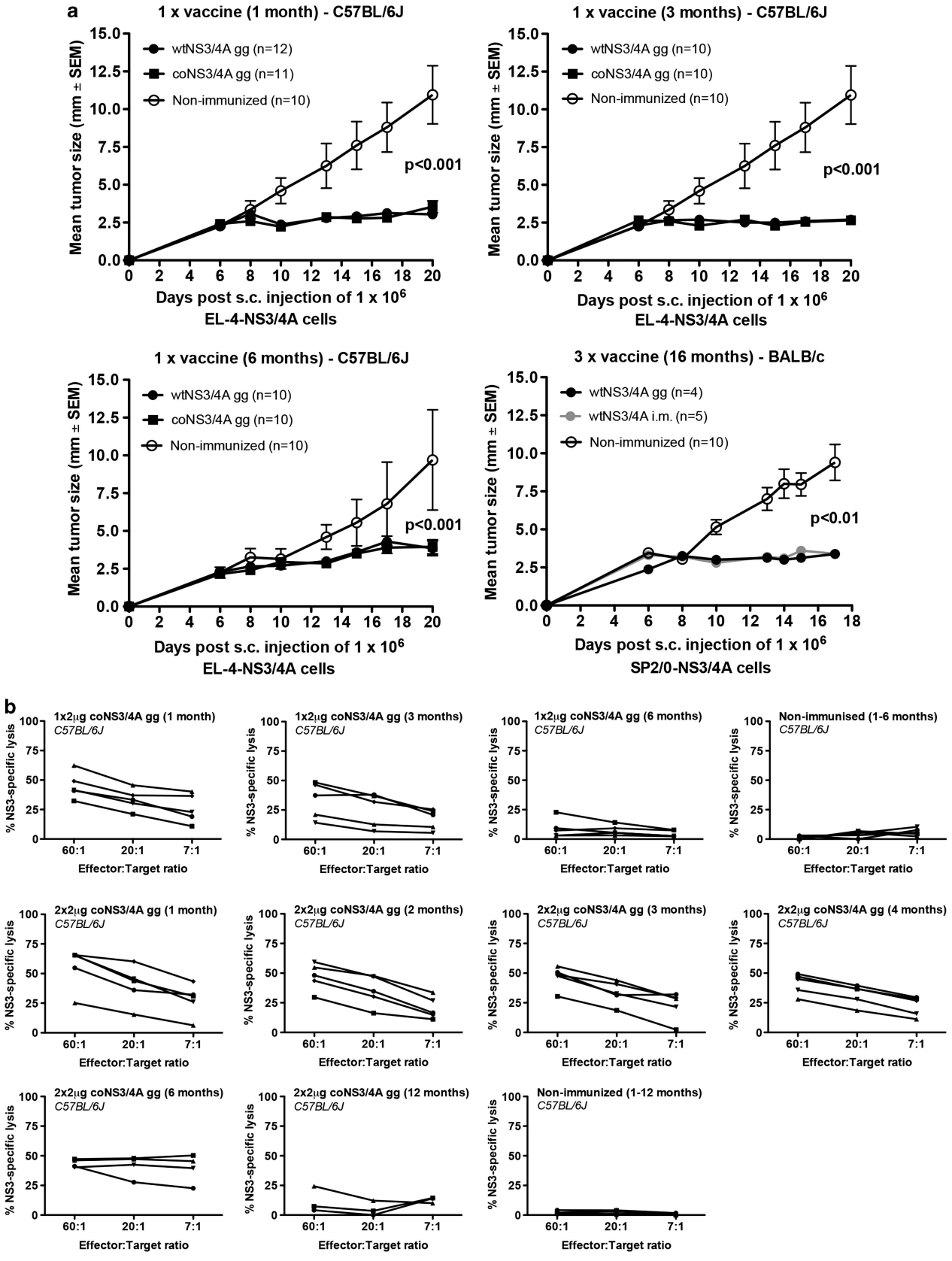
Next, we were interested in testing the specificity of the murine H-2^b CTL epitope. Hence, we compared mice immunized transdermally two times using wtNS3/4A DNA vaccines containing the wt sequence (GAVQNEVTL) or a mutated sequence where positions 5 and 9 had been changed to alanine instead of an asparagine and leucine, respectively (GAVQAEVTA). Two weeks after the last immunization, mice were killed and the cytolyic activity was monitored using target cells presenting the wt sequence. We showed that only mice immunized with a DNA vaccine containing the wt sequence demonstrated T cells with lytic activity (Figure 5a). By mutating positions 5 and 9, we markedly affected the ability of the peptide to bind the MHC H-2D^b molecule (Figure 5b). Also, the single mutations markedly affected the MHC-peptide-binding affinity and it was only the wt peptide that had a strong binding affinity for H-2D^b (Figure 5b). Polymorphism within the GAVQNEVTL epitope was rare with only one mutation at position 5 and none at position 9 found in 40 published GenBank isolates (Supplementary Figure 3). Next, we were interested in whether DNA immunization using the wtNS3-/4A or mutated DNA vaccine could protect mice from tumor growth. Specifically, we immunized groups of 10 C57BL/6J mice once by gene gun with 2 μg of indicated DNA vaccine. Control mice were left non-immunized. Two weeks after immunization, mice were challenged with a subcutaneous inoculation of 1×10^6 NS3/4A-EL-4 cells, and tumor growth was monitored for up to 3 weeks (Figure 5c). Our results show that wtNS3/4A-based vaccination mediated protection against tumor growth ($P < 0.01$), whereas wtNS3/4AΔ5,9-based vaccine only mediated a partial protection ($P =$ NS; Figure 5c). There was no statistically significant difference between groups of mice immunized with wtNS3-/4A or wtNS3/4AΔ5,9 vaccines ($P =$ NS; Figure 5c).

DISCUSSION

We designed an in-depth study to evaluate the induction of HCV NS3/4A-specific long-term memory responses after genetic immunization in two mouse strains. Previous studies evaluating the longevity of immune responses analyzed the induced immune responses approximately 3–6 months after the last immunization.[25–28] Hence, there is a lack of understanding of the abilities to prime long-term memory T-cell responses by genetic vaccines. We therefore extended the analysis of immune responses from 2 weeks to 12–16 months after the last immunization. In addition, we also determined differences in immune activation using different NS3- and NS3/4A-based vaccine constructs, administration routes and mouse haplotypes and strains (e.g. BALB/c and wt and IFNγR2−/− C57BL/6J mice). Notably, the NS3/4A DNA vaccine has already been tested in a phase I/II clinical trial for safety, immunogenicity and effects on viral load in chronic HCV patients with genotype 1 infection.[15] In the clinical trial, the longevity of NS3/4A-specific T-cell responses was not analyzed beyond 24 weeks after the last immunization. Interestingly, we found NS3/4A-specific T-cell responses in 3 out of 12 patients at week 24 after the last immunization.[15]

To be able to monitor CD8+ T-cell responses after NS3/4A DNA immunization in mice, MHC class I CTL epitopes had to be identified. We used the H-2^b and H-2^d mouse strains for monitoring NS3/4A-specific T-cell responses. We have previously identified an H-2D^b restricted NS3 epitope to which NS3/4A-specific T cells are targeted.[17,22] For the H-2^d mouse strain (e.g. BALB/c), no CD8+ T-cell epitope has been described. Thus, from a

Figure 2. Evaluation of the ability of different immunogens to prime HCV NS3/4A-specific tumor-inhibiting responses after DNA immunization. Groups of 8–10 C57BL/6J or BALB/c mice were either left untreated or were given one (**a**), two or four monthly (**b**) gene gun (gg) immunizations of 2 μg plasmid DNA of the indicated immunogens. At 2 weeks after the last immunization, mice were subcutaneously inoculated with 1×10^6 NS3/4A-expressing EL-4 (**a**) or NS3/4A-expressing SP2/0-Ag14 (**b**) cells. Tumor sizes were measured through the skin at days 6–19 after tumor inoculation. Values are given as the mean tumor size ± s.e.m. Also given is the *P*-value obtained from the statistical comparison of immunized and control groups using the area under the curve (AUC) and analysis of variance. In (**c**), the NS3-specific lytic activity after two immunizations with indicated immunogens in groups of five C57BL/6 and IFNγR2−/− mice are shown. In (**d**), the percentage of IFN-γ- or TNFα-producing CD8$^+$ T cells in groups of five immunized C57BL/6 mice (five mice per pool) with indicated immunogens. In (**e** and **f**), representative dot plots from each group of mice showing the IFN-γ- and TNFα-positive CD8$^+$ T cells.



set of 68 20-mer peptides covering the NS3/4A region, we identified one H-2K^d-restricted NS3 epitope (e.g. 10-mer, amino-acid sequence: AYMNTPGLPV). This epitope binds the H-2K^d molecule and induces NS3-specific CTL responses in BALB/c mice after immunization as a peptide or DNA vaccine. Hence, the identified H-2^d epitope was shown to induce similar levels of lytic activity as the previously described NS3 CTL epitope in H-2^b mice.[17,22] In addition, the specificity of the epitope was restricted to H-2^d mice as no lytic activity was recorded to the epitope in H-2^b mice, but clearly detected in outcrossed H-2^{d}xH-2^b mice. We have previously shown that both prophylactic and therapeutic vaccination using NS3/4A-based DNA vaccines mediates complete or partial protection against *in vivo* challenge using syngeneic tumor cells expressing the NS3/4A proteins.[17] Our results confirm the importance of including NS4A in NS3-based vaccines for improved immunogenicity. The most potent immune activation was obtained when immunizing mice with the full-length NS3/4A-pVAX1 construct, especially when the construct was codon optimized. Importantly, this construct primed multifunctional T-cell responses evidenced by the production of IFN-γ or TNFα in addition to potent lytic activity. Activation of multifunctional T-cell responses to NS3/4A has been shown previously using the coNS3/4A-pVAX1 construct.[29] The superiority of the coNS3/4A-pVAX1 construct in priming NS3/4A-specific immune responses was also replicated in the cytotoxicity assay showing the highest lytic activity in mice immunized with coNS3/4A followed by the wtNS3/4A and wtNS3/4AΔRGT, which encodes a dysfunctional NS3 protease. One may argue that a vaccine with a dysfunctional NS3 protease has a better safety profile, as the functional NS3 protease is known to interfere with several host proteins.[30] However, the recently evaluated coNS3/4A-pVAX1 vaccine, encoding a functional protease, was found safe and well tolerated in a phase I/IIa clinical trial.[15]

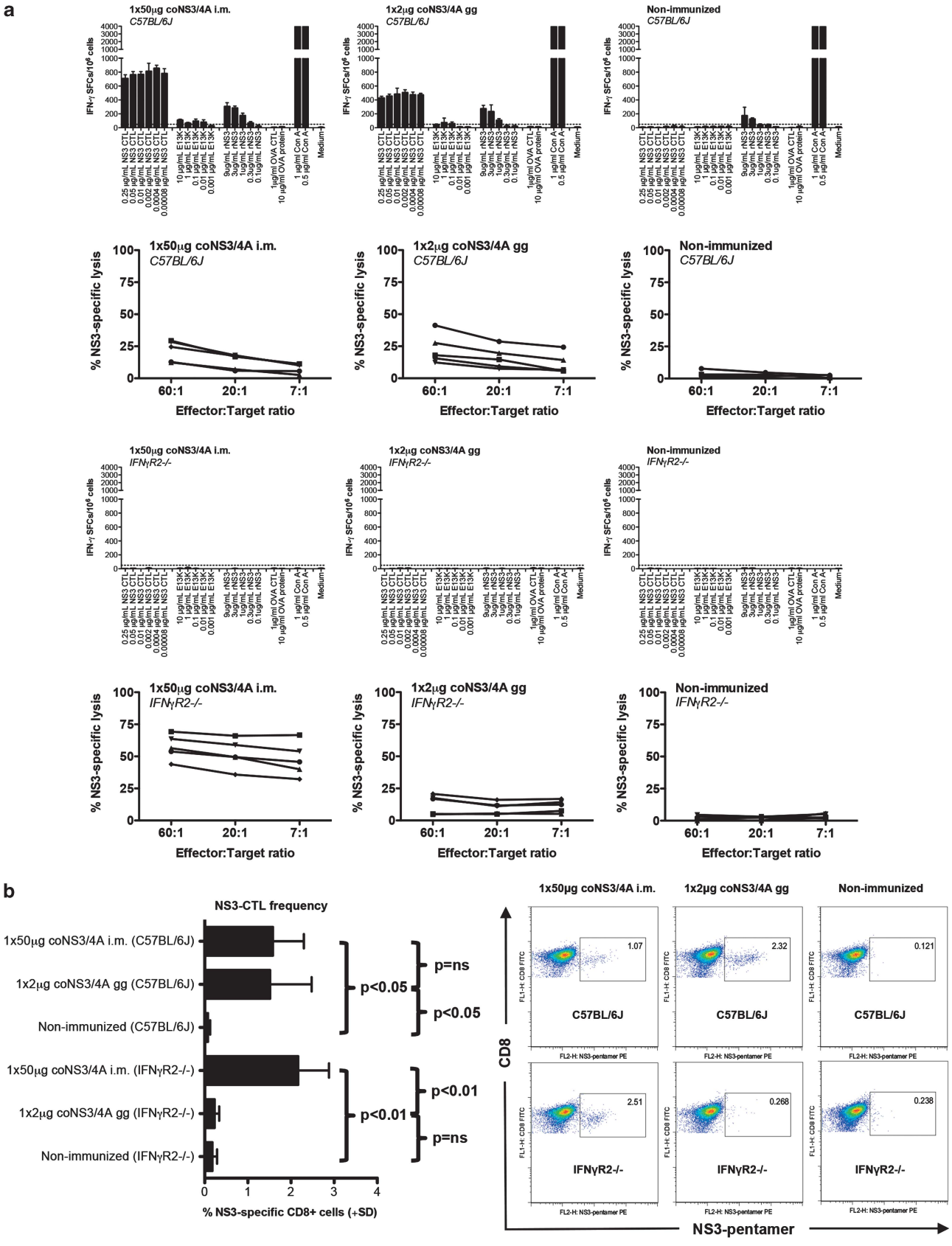
A key question in the current study was how long after the last immunization could activated HCV-specific T cells perform their effector functions. We showed that in addition to mediating protection 2 weeks after the last immunization, significant tumor inhibition occurred after 1, 3, 6 and 16 months. These impressive results were confirmed by determining the *in vitro* lytic activity at similar time points after immunization. Hence, both tumor protection and lytic activity showed similar results of long-term protective immune responses.

In addition, we were interested in the importance of IFN-γ signaling during immune activation. Hence, we immunized wt and IFNγR2 − / − mice with the coNS3/4A construct and thereafter analyzed the cytolytic activity. Mice lacking the IFNγR2 chain are known to be unresponsive to IFN-γ.[31,32] Our data show that the IFNγR2 − / − mice, even though unresponsive to IFN-γ, demonstrated lytic NS3/4A-specific CTLs. However, IFNγR2 − / − mice have been shown to be unable to clear NS3/4A protein expression in hepatocytes.[33] One explanation for this finding is that NS3/4A-specific CTLs fail to enter the liver, which is consistent with data showing that IFN-γ is involved in trafficking of T cells to infected sites.[34] Next, we were interested in any differences in immune activation between intramuscular and transdermal delivery of the coNS3/4A DNA vaccine in wt and IFNγR2 − / − mice. No significant differences were found in wt mice immunized by the intramuscular or the transdermal route determined by IFN-γ ELISpot, cytotoxicity assay and quantification of NS3-specific CTLs. On the other hand, when immunizing IFNγR2 − / − mice, major differences were detected. In particular, activation of lytic CTLs and the frequency of NS3-specific CTLs were significantly reduced in the transdermal group compared with the intramuscular group. This may be explained by the different routes of immunization, where the intramuscular route target mainly myocytes, whereas the transdermal route mainly targets Langerhans cells and other professional antigen-presenting cells. We have previously shown that the coNS3/4A DNA vaccine induced a T-helper type 1 skewing of the T-cell response after intramuscular immunization, whereas a transdermal immunization induced a mixed T-helper type 1/T-helper type 2 response.[17] Also, other factors such as the antigen dose and level of costimulation may be involved. This merits further investigation.

The importance of homologous gene sequences in the vaccine and the infecting virus were investigated to determine if genotype-specific vaccines are needed to control or clear HCV infections. Therefore, H-2^b mice were immunized with either a wtNS3/4A DNA vaccine or a vaccine with alanine substitutions at positions 5 and 9 in the murine epitope (GAVQNEVTL to GAVQAEVTA). We found that mutations in positions 5 and 9 in the NS3 CTL epitope completely abolished the induction of lytic T cells to NS3. This could be explained by a complete loss of MHC peptide binding for the mutated epitope. Hence, our results show the importance of using vaccines with similar gene sequences as the infecting virus. Similar data have been shown for the immune-dominant HLA-A2-restricted NS3-specific 1073–1081 CTL epitope.[35] However, certain mutations within the 1073–1081 CTL epitope have been shown to have effects on the viral fitness. Thus, such mutations may never be found in the infected host. In addition, mutations within the 1073–1081 CTL epitope may render the virus resistant to NS3 DAAs, but it may still be a target for NS3/4A vaccine-primed CTLs.[36] To investigate the importance of the GAVQNEVTL epitope *in vivo*, mice were immunized with the wtNS3/4A or mutated DNA vaccines and the tumor-inhibiting immune responses were determined. The wtNS3/4A DNA vaccine conferred protection against tumor growth, whereas the mutated DNA vaccine only mediated a partial protection. This highlights that not only the GAVQNEVTL epitope is of importance but also additional epitope/s for obtaining tumor-protective immune responses *in vivo*.

In summary, the current study demonstrates for the first time that an HCV-based DNA vaccine can induce long-term memory T-cell responses, which are detectable in mice up to 12–16 months after the last immunization. In addition, we identified a new murine H-2^d CTL epitope that enables detailed monitoring of NS3-specific immune responses in BALB/c mice. Taken together, the data obtained indicate that NS3/4A should be included in HCV vaccine compositions.

Figure 3. Evaluation of the longevity of HCV NS3/4A-specific tumor-inhibiting responses. Protection against tumor growth was evaluated 1, 3, 6 or 16 months after one or three DNA immunizations (**a**). Groups of 4–12 C57BL/6J mice were either left untreated or were given one (gene gun (gg) delivery) or three (gg or intramuscular delivery) immunizations of 2 μg wtNS3/4A-pVAX1 or coNS3/4A-pVAX1 plasmid DNA. A dose of 100 μg wtNS3/4A-pVAX1 was used for the intramuscular immunizations. Tumor sizes were measured through the skin at days 6–20 after tumor inoculation. Values are given as the mean tumor size ± s.e.m. Also given is the *P*-value obtained from the statistical comparison of immunized and control groups using the AUC and analysis of variance. In (**b**), the presence of lytic T-cell responses was determined at 1, 3 and 6 months after a single transdermal gg immunization and at 1, 2, 3, 4, 6 and 12 months after two monthly transdermal gg immunizations with 2 μg coNS3/4A-pVAX1. Each group consisted of five mice. Two weeks after the last immunization, specific lytic activity was determined using peptide-loaded RMA-S cells at E:T ratios of 60:1, 20:1 and 7:1 in a standard ^{51}Cr-release assay. Specific lysis above 10% was considered positive. Each line indicates an individual mouse.



MATERIALS AND METHODS

Animals

Inbred female BALB/c (H-2^d), C57BL/6J (H-2^b), C57BL/6JxBALB/c (H-2^{d}xH-2^b) and IFNγR2-deficient (e.g. IFNγR2 − / −, H-2^b) mice were housed at Karolinska Institutet, Division of Comparative Medicine, Clinical Research Center, Karolinska University Hospital, Huddinge, Sweden. The animals were either purchased (Charles River Laboratories, Sulzfeld, Germany) or bred in-house. The animals were caged (5–10 mice per cage) and fed with a commercial diet (RM3 (p) PL IRR diet; Special Diet Service) with free access to food and water. All animals were 6–10 weeks of age at the start of the experiment. All experimental protocols involving animals were approved by the Ethical Committee for Animal Research at Karolinska Institutet.

Cell lines

The SP2/0-Ag14 and SP2/0-NS3/4A myeloma cell lines (H-2^d)[22] was maintained in Dulbecco's modifed Eagle's medium medium supplemented with 10% fetal bovine serum (FBS) (Sigma Aldrich, St Louis, MO, USA), 2 mM L-glutamine, 10 mM HEPES, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 1 mM nonessential amino acids, 50 μM β-mercaptoethanol and 1 mM sodium pyruvate (Sigma Aldrich). SP2/0-Ag14 cells with stable expression of NS3/4A were maintained in 800 μg geneticin (G418) per ml complete Dulbecco's modifed Eagle's medium.

The EL-4 and EL-4-NS3/4A lymphoma cell lines (H-2^b)[17] were maintained in RPMI 1640 medium supplemented with 10% FBS, 10 mM HEPES, 1 mM sodium pyruvate, 1 mM nonessential amino acids, 50 μM β-mercaptoethanol, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Sigma Aldrich). EL-4 cells with stable expression of NS3/4A were maintained in 800 μg geneticin (G418) per ml complete RPMI 1640 medium.

RMA-S cells (H-2K^d, H-2D^d or H-2L^d) were maintained in RPMI 1640 medium supplemented with 5% FBS, 2 mM L-glutamine, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Sigma Aldrich). All cells were grown in a humidified 37 °C and 5% CO_2 incubator.

Plasmid DNA

The following plasmids: wtNS3-pVAX1, wtNS3/4A-pVAX1 and coNS3/4A-pVAX1 have been described previously and originate from an HCV genotype 1a virus[17,22] (GenBank accession number: AR820945.1; http://www.ncbi.nlm.nih.gov/genbank, ChronVac-C; ChronTech Pharma

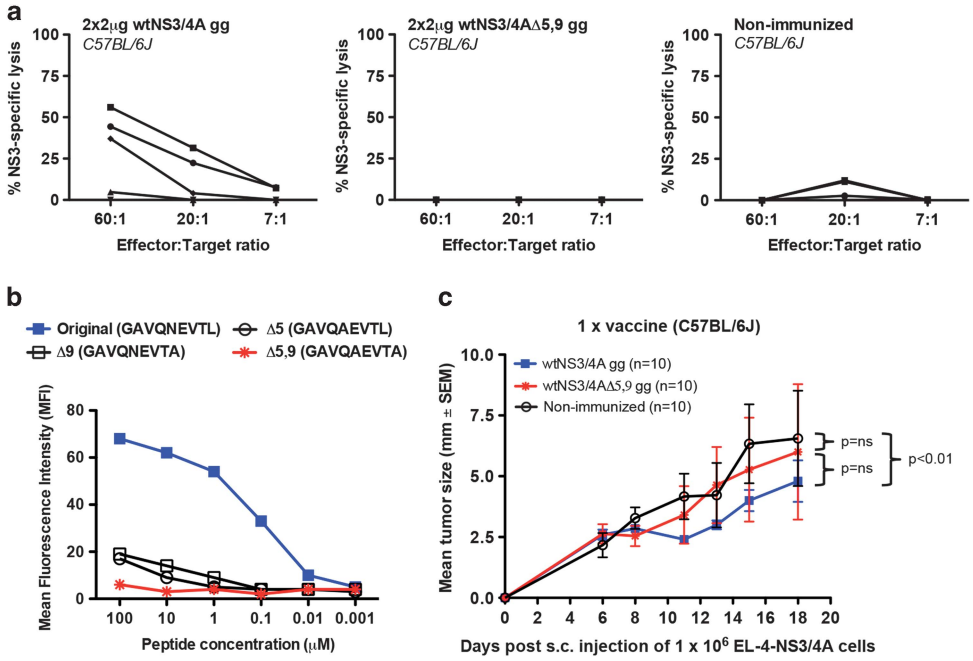


Figure 5. Evaluation of *in vitro* detectable CTLs and tumor-inhibiting responses in C57BL/6J mice immunized with wtNS3/4A-pVAX1 or wtNS3/4AΔ5,9-pVAX1 plasmid DNA. (**a**) Groups of five C57BL/6J mice were immunized once with 2 μg plasmid DNA using transdermal gene gun (gg) delivery or were left untreated (non-immunized). Two weeks after the last immunization, the NS3-specific lytic activity was determined using peptide-loaded (GAVQNEVTL) RMA-S cells at E:T ratios of 60:1, 20:1 and 7:1 in a standard ^{51}Cr-release assay. Specific lysis above 10% was considered positive. Each line indicates an individual mouse. In (**b**), the *in vitro* peptide stabilization of MHC–peptide complexes on transporter associated with antigen processing 2-deficient RMA-S cells transfected with H-2D^b is shown. Binding affinities were determined by flow cytometry measuring the mean fluorescence intensity (MFI) for each peptide in descending concentrations. (**c**) Groups of 10 C57BL/6J mice were either left untreated or were given one gg immunizations of 2 μg wtNS3/4A or wtNS3/4AΔ5,9 plasmid DNA. At 2 weeks after immunization, mice were subcutaneously inoculated with 1 × 10^6 NS3/4A-expressing EL-4 cells. Tumor sizes were measured through the skin at days 6–18 after tumor inoculation. Values are given as the mean tumor size ± s.e.m. Also given is the *P*-value obtained from the statistical comparison of wtNS3/4A and non-immunized or wtNS3/4AΔ5,9 and non-immunized or wtNS3/4A and wtNS3/4AΔ5,9 using the AUC and analysis of variance. NS, not significant.

Figure 4. Priming of NS3-specific immune responses after intramuscular and transdermal DNA immunization. Groups of five C57BL/6J or IFNγR2 − / − mice were immunized using intramuscular needle injection, transdermal gene gun (gg) injection or left untreated. At 2 weeks after the last immunization, mice were killed and splenocytes harvested for determination of T-cell responses. In (**a**, rows 1 and 3), the number of IFN-γ spot-forming cells (SFCs) by ELISpot assay was determined 36 h after *in vitro* stimulation of splenocytes with CTL and T-helper peptides or recombinant NS3 protein at the indicated concentrations. Results are given as mean SFCs per 10^6 (± s.d.), the cutoff was set to 50 SFCs per 10^6 splenocytes. In (**a**, rows 2 and 4), the lytic activity determined by a ^{51}Cr-release assay using peptide-loaded (GAVQNEVTL) RMA-S cells from individual mice at E:T ratios of 60:1, 20:1 and 7:1 is shown. Specific lysis above 10% was considered positive. Each line indicates an individual mouse. In (**b**), expansion of NS3-specific CD8$^+$ T cells was determined using direct *ex vivo* pentamer staining. GAVQNEVTL epitope-specific CD8$^+$ T-cell frequencies are shown as the percentage of GAVQNEVTL pentamer-positive CD8$^+$ T cells (± s.d.). Also, representative dot plots from each group are shown. The presence of a statistical difference (Mann–Whitney *U*-test) was indicated as follows: $P < 0.05$ and $P < 0.01$. NS, not significant.

AB, Huddinge, Sweden). The coNS3/4A plasmid contain an NS3/4A gene sequence that have been codon optimized by adjusting the codon usage to the codons most commonly used by human cells. This was carried out to improve the protein expression levels of NS3/4A.[17] Additionally, a wtNS3/4AΔ5,9-pVAX1 (GAVQAEVTA) plasmid with alanine substitutions at positions 5 and 9 in the previously identified CTL epitope GAVQNEVTL[22] was generated by QuikChange Site-Directed Mutagenesis according to the manufacturer's protocol (Agilent Technologies, Santa Clara, CA, USA). In the same way, a wtNS3/4AΔRGT-pVAX1 plasmid encoding a dysfunctional NS3 protease was generated.

Plasmids were grown in competent TOP10 *Escherichia coli* (Life Technologies, Carlsbad, CA, USA) and purified using Qiagen DNA purification columns according to the manufacturer's instructions (Qiagen, Hilden, Germany). Plasmid DNA concentration was determined spectrophotometrically and the purified DNA was dissolved in sterile phosphate-buffered saline (PBS) at concentrations of 1 mg ml^{-1}. Restriction enzyme digests were performed to ensure that the plasmid contained the gene of interest with the correct size. All DNA plasmids were sequenced to ensure correct nucleotide sequence (Eurofins MWG Operon, Ebersberg, Germany).

Peptides and proteins

Twenty-mer and 10-mer peptides (Supplementary Figure 1) derived from the full-length genotype 1a NS3/4A protein and the peptides (GAVQNEVTL, aa 1629–1637 (ref. 22) and AYMNTPGLPV, aa 1541–1550) corresponding to the NS3 CTL epitopes in H-2D^b and H-2K^d mice, respectively, EIPFYGKAI-PLEAIK (NS3-Th E13K gt1a) NS3 mouse class II epitope (aa 1372–1386)[29] and SIINFEKL (OVA 257–264, CTL) from ovalbumin (OVA) was synthesized by automated peptide synthesis as described previously (ChronTech Pharma AB).[37] Recombinant NS3 protein gt1a (aa 1207–1612) were produced in *E. coli* and purified as described.[38] Chicken egg albumin (OVA) and concanavalin A were purchased from Sigma Aldrich.

Immunization protocol

Groups (4–12 mice per group) of female C57BL/6J, BALB/c, C57BL/6J × BALB/c or IFNγR2 − / − mice, 6–10 weeks old, were immunized by needle injections of 100 μg of plasmid DNA encoding HCV NS3/4A. Plasmid DNA in PBS was given intramuscularly in the *tibialis cranialis* muscle. Mice were boosted at monthly intervals. For gene gun-based immunizations (e.g. transdermal immunization), plasmid DNA was linked to gold particles (1 μm) according to the manufacturer's protocol (Bio-Rad, Hercules, CA, USA) and as described in detail.[39] Before immunization, the abdominal immunization area was shaved and the immunization was performed at a helium discharge pressure of 500 psi. Each injection dose contained 2 μg of plasmid DNA. The mice were boosted with the same dose at monthly intervals.

ELISpot assay

Spleen cells from groups of mice (5 mice per group) were pooled and tested for the presence of NS3/4A-specific T cells. The ability of NS3/4A-specific CTLs and T-helper cells to produce IFN-γ and IL-2 after exposure to different peptides: GAVQNEVTL (NS3-CTL) sequence originates from NS3 (H-2D^b; aa 1629–1637), EIPFYGKAIPLEAIK (NS3-Th E13K gt1a) sequence originates from NS3 that contain a mouse class II epitope (aa 1372–1386) and recombinant NS3 protein 1a (aa 1207–1612), SIINFEKL (CTL, OVA 257–264), proteins (OVA grade VII; Sigma Aldrich), concanavalin A (Sigma Aldrich) and media were also assessed. Production of IFN-γ and IL-2 cytokines was determined by a commercially available ELISpot assay. In brief, ELLIP plates (Merck Millipore, Billerica, MA, USA; cat. no MAIPSWU) with polyvinylidene difluoride membranes were treated with 70% ethanol for 1 min, washed in sterile water and coated overnight at +4 °C with 10 μg ml^{-1} of monoclonal antibodies specific for IFN-γ (AN18) or IL-2 (1A12) (Mabtech, Nacka Strand, Sweden) in PBS. After washing five times in PBS, the plates were blocked for 2 h with complete RPMI 1640 medium. All stimulations (24–48 h at 37 °C, 5% CO_2) were carried out using 200 000 splenocytes per well. Various concentrations of the different antigens were added in triplicate to a total volume of 200 μl. After stimulation, the wells were washed and incubated for 2 h at 37 °C with biotinylated antibodies, respectively: anti-IFN-γ (R4–6A2-biotin) and anti-IL-2 (5H4-biotin) (Mabtech) at 2 μg ml^{-1} in 0.5% FBS/PBS (Sigma Aldrich). After washing, Strep-ALP (Mabtech) diluted 1:1000 in 0.5% FBS/PBS was added and incubated for 1 h in room temperature. Sterile-filtered substrate, BCIP/NBT (5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium) (Mabtech), was used to develop spots: IFN-γ for 12 min and IL-2 for 15 min. The substrate reaction was stopped by rinsing extensively with dH_2O, after which the plates were left to dry. The number of spots was counted using the AID iSpot reader and software ver. 7.0 (AID, Strassberg, Germany). The number of spots (cytokine-producing cells) was determined at each concentration of peptide or protein and the results given as the number of IFN-γ or IL-2-producing cells per 10^6 cells. A mean number of cytokine-producing cells of 50 per 10^6 cells were considered as negative.

Detection of NS3/4A-specific CTLs by cytotoxicity assay (e.g. ^{51}Cr-release assay)

Spleens from DNA-immunized mice were collected 2 weeks after the last immunization and single-cell suspensions were prepared in RPMI 1640 medium supplemented with 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Sigma Aldrich). Red blood cells were removed using Red Blood Cell Lysing Buffer (Sigma Aldrich). Splenocytes were stimulated *in vitro* by cocultivation in 25 ml flasks containing 12 ml complete RPMI 1640 medium for 5 days with an equal number of irradiated (2000 rad) syngeneic splenocytes and an MHC class I-specific peptide at a concentration of 0.05 μM. Effector cells were harvested at day 5 and a ^{51}Cr-release assay was performed in V-bottomed 96-well plates. As target cells, peptide-loaded RMA-S cells were used. The RMA-S target cells were incubated with specific MHC class I HCV NS3 peptide H-2D^b (GAVQNEVTL) or H-2K^d (AYMNTPGLPV) peptide at a concentration of 50 μM for 90 min at 37 °C in a 5% CO_2. The cells were carefully mixed every 15 min. Target cells were incubated for 1 h at 37 °C with 30 μl ^{51}Cr (5 mCi ml^{-1}; PerkinElmer, Waltham, MA, USA) and washed three times in PBS before use. Target cells were added to the plates in a total volume of 200 μl at effector:target (E:T) ratios of 60:1, 20:1 and 7:1. The cytotoxic activity was determined after a 4 h incubation at 37 °C in a 5% CO_2. Twenty-five microliters of supernatant were harvested and transferred to 200 μl OptiPhase SuperMix (PerkinElmer) in a 96-well isoplate (PerkinElmer), and the radioactivity was detected using a 1450 Microbeta Trilux scintillation counter, with the Microbeta Workstation Software 4.0 (PerkinElmer). The percent of specific ^{51}Cr release was calculated according to the formula: (experimental release − spontaneous release)/(maximum release − spontaneous release) x100. Experimental release is the mean count per minute of ^{51}Cr released by target cells in the presence of effector cells. Maximum release was calculated from supernatants of cells that were lysed. Spontaneous release was calculated from supernatants of cells incubated without effector cells. All samples were run in triplicate.

Quantification of NS3-specific CD8+ T cells by pentamer staining

The frequency of NS3-specific CD8+ T cells was analyzed by direct *ex vivo* staining of splenocytes using the NS3 GAVQNEVTL genotype 1a Pro5 pentamer exactly as described previously.[21]

In vivo challenge with the NS3/4A-expressing tumor cells

The tumor challenge model is a robust model for testing vaccine efficacy; however, it also has limitations because it does not take into account viral evolution and possible occurrence of viral escape mutations. *In vivo* challenge of immunized mice with the NS3/4A-expressing SP2/0-Ag14 myeloma or EL-4 lymphoma cell lines were performed according to the method described previously.[17,22,39] In brief, groups of BALB/c or C57BL/6J mice were immunized with different immunogens at monthly intervals. At 2 weeks to 16 months after the last immunization, 1 × 10^6 NS3/4A-expressing SP2/0-Ag14 or EL-4 cells were subcutaneously inoculated in the right flank. The flanks were shaved before inoculation. The kinetics of tumor growth was determined by measuring the tumor size of the tumor through the skin at days 6–20. The kinetics of tumor development in groups of mice was compared using the area under the curve (AUC). The mean tumor sizes were compared using the analysis of variance test. Latest at day 20, all mice were killed.

Detection of intracellular IFN-γ and TNFα-production

Spleen cells from groups of immunized or non-immunized mice were pooled and tested for the presence of intracellular IFN-γ and TNFα production after *in vitro* peptide stimulation with. The *in vitro* stimulation was carried out in U-bottom 96-well plate at a cell density of 0.75 × 10^6 cells per 200 μl per well. The cells were cultured in the presence of 0.2 μl per well of Golgi plug (Becton Dickinson (BD) Biosciences, Franklin Lakes, NJ, USA). The final concentration of each peptide was 12.5 μM. Cells were stimulated with phorbol

12-myristate 13-acetate (Sigma Aldrich) at 0.005 μg ml^{-1} and ionomycin (Sigma Aldrich) at 0.5 μg ml^{-1} were include as a positive control. After a 4-h incubation, cells were washed two times in FACS buffer and preincubated with Fc block. Cells were thereafter stained for the cell surface markers (fluorescein isothiocyanate-conjugated anti-mouse CD8 (clone 53–6.7), CyChrome-conjugated anti-mouse CD4 (clone RM4-5) and CyChrome-conjugated anti-mouse B220 (clone RA3-6B2) (BD Biosciences)). Cells were then washed two times in FACS buffer, fixed and permeabilized with Cytofix/Cytoperm solution (BD Biosciences) according to the manufacturer's instructions. Cells were then stained with phycoerythrin-conjugated anti-mouse IFN-γ (clone XMG1.2) antibody (0.8 μg per 10^6 cells) or phycoerythrin-conjugated anti-mouse TNFα antibody (0.8 μg per 10^6 cells) diluted in Perm/Wash solution (BD Biosciences) for 30 min on ice, and then washed and analyzed on a FACS Calibur (BD Biosciences). Generally, 100 000 total events were acquired and data were analyzed using the CellQuest software (BD Biosciences).

RMA-S stabilization assay

Peptides were evaluated for the ability to stabilize the MHC class I molecule. The RMA-S stabilization assay was performed exactly as described previously.[40] Antibodies against mouse H-2K^d, H-2D^d and H-2L^d was used to detect stabilized MHC class I molecules. The following antibodies were purchased from BD Biosciences: fluorescein isothiocyanate-conjugated H-2K^d (clone SF1-1.1) and H-2D^d (clone 34-2-12), phycoerythrin-conjugated anti-mouse IFN-γ (clone XMG1.2) and fluorescein isothiocyanate-conjugated anti-mouse CD8 (clone 53–6.7), CyChrome-conjugated CD4 (clone RM4-5) and CyChrome-conjugated B220 (clone RA3-6B2), and anti-CD16/CD32 (Fc block, 2.4G2). The anti-H-2L^d (clone 30-5-7s) supernatant was kindly provided by Professor Klas Kärre (Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Sweden). Fluorescein isothiocyanate-conjugated goat anti-mouse Ig (Dakopatts, Hellerup, Denmark) were used as secondary reagent for the H-2L^d antibody. Data were acquired on a FACS Calibur (BD Biosciences) and presented as mean fluorescence intensity.

Statistical analysis

All comparisons were performed using GraphPad InStat 3, Macintosh (version 3.0b, 2003; GraphPad Software, San Diego, CA, USA) and Microsoft Excel 2011, Macintosh (version 14.3.9; Microsoft, Redmond, WA, USA). Kinetic measurements were compared using the AUC (Excel). Parametrical data were compared using the analysis of variance and non-parametrical data with Mann–Whitney *U*-test.

CONFLICT OF INTEREST

LF owns patent/patents pending for HCV-transgenic mouse models and HCV therapeutics. The remaining authors declare no conflict of interest.

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