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Enhanced and Sustained CD8⁺ T Cell Responses with an Adenoviral Vector-Based Hepatitis C Virus Vaccine Encoding NS3 Linked to the MHC Class II Chaperone Protein Invariant Chain

Marianne Mikkelsen,* Peter Johannes Holst,* Jens Bukh,*‡ Allan Randrup Thomsen,* and Jan Pravsgaard Christensen*

Potent and broad cellular immune responses against the nonstructural (NS) proteins of hepatitis C virus (HCV) are associated with spontaneous viral clearance. In this study, we have improved the immunogenicity of an adenovirus (Ad)-based HCV vaccine by fusing NS3 from HCV (Strain J4; Genotype 1b) to the MHC class II chaperone protein invariant chain (Ii). We found that, after a single vaccination of C57BL/6 or BALB/c mice with Ad-IiNS3, the HCV NS3-specific CD8⁺ T cell responses were significantly enhanced, accelerated, and prolonged compared with the vaccine encoding NS3 alone. The AdIiNS3 vaccination induced polyfunctional CD8⁺ T cells characterized by coproduction of IFN-γ, TNF-α and IL-2, and this cell phenotype is associated with good viral control. The memory CD8⁺ T cells also expressed high levels of CD27 and CD127, which are markers of long-term survival and maintenance of T cell function. Functionally, the AdIiNS3-vaccinated mice had a significantly increased cytotoxic capacity compared with the AdNS3 group. The AdIiNS3-induced CD8⁺ T cells protected mice from infection with recombinant vaccinia virus expressing HCV NS3 of heterologous 1b strains, and studies in knockout mice demonstrated that this protection was mediated primarily through IFN-γ production. On the basis of these promising results, we suggest that this vaccination technology should be evaluated further in the chimpanzee HCV challenge model. The Journal of Immunology, 2011, 186: 2355–2364.

Persistent hepatitis C virus (HCV) infection is a major health problem worldwide; it affects ~3% of the world’s population and is an important contributor to chronic liver disease (1). Globally, HCV exists as seven major genotypes and over 50 subtypes. In the Western world genotype 1 is the most prevalent and also the most difficult HCV genotype to treat with the available IFN-based therapy (2). Yearly, there are 3–4 million new HCV infections worldwide. However, only 10–25% of acutely infected individuals resolve HCV during the first 6 mo, and the majority develops persistent lifelong infections (3). Development of effective prophylactic and therapeutic vaccines against HCV are therefore of high priority, because they could significantly lower the chronicity rate, and thus have a major influence on the disease burden.

*Department of International Health, Immunology and Microbiology, Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark; and Copenhagen Hepatitis C Program, Department of Infectious Diseases and Clinical Research Centre, Copenhagen University Hospital, Hvidovre, Denmark.

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Address correspondence and reprint requests to Jan Pravsgaard Christensen, Department of International Health, Immunology and Microbiology, University of Copenhagen, The Panum Institute, Building 22.5.12, DK-2200 Copenhagen N, Denmark. E-mail address: jpc@sund.ku.dk.

Abbreviations used in this article: Ad, adenovirus; HCV, hepatitis C virus; Ii, MHC class II chaperone protein invariant chain; KO, knockout; LCMV, lymphocytic choriomeningitis virus; NS, nonstructural; p.v., post vaccination; TCM, central memory T cell.

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The fact that one of four HCV infected individuals spontaneously resolve their HCV infection gives hope for successful development of an effective vaccine. The factors of importance for spontaneous clearance of HCV are not fully understood, but studies of HCV-infected patients and experimentally infected chimpanzees, the only other species susceptible to HCV infection, suggest that vigorous, multispecific and sustained memory T cell responses have an important role (4). Strong, functional, and multispecific CD4⁺ T cell responses and a broad cytotoxic CD8⁺ T cell response against the nonstructural (NS) proteins of HCV are associated with self-limited HCV infection (5–9). In addition, the importance of both CD4⁺ and CD8⁺ memory T cells for protection against persistent HCV infection has been demonstrated by depletion studies performed in experimentally infected chimpanzees (10, 11). The significance of the cellular immune response is further underscored by the fact that experimentally infected chimpanzees were found to be protected against repeated rechallenges in the absence of neutralizing Abs (12). Studies of other chronic viral infections indicate that, in addition to the frequency and specificity of the CD4⁺ and CD8⁺ T cells, the ability of the virus-specific memory cells to simultaneously produce multiple antiviral cytokines (IFN-γ, TNF-α, and IL-2) might be associated with good viral control (13–15).

Several HCV vaccine studies have been conducted in both mice and chimpanzees using various vaccine approaches, such as E1E2 glycoprotein vaccination, vaccination with viral like particles, DNA vaccinations targeting structural and NS proteins, and viral vectors targeting NS proteins (16). Despite strong evidence for the importance of cellular immunity in the combat against HCV infection, it has been a major challenge to design potent vaccines against HCV. The frequency of T cells directed toward the NS proteins of HCV observed in HCV vaccine studies range from 0.3% after vaccination with NS3/NS4A vaccine as measured after
in vitro stimulation for 5–7 d to 0.9% of the CD8+ T cell population after three vaccinations with Th1-adjuvanted polyepitopes and defective alpha-viral particles measured by ex vivo intracellular IFN-γ production (17–20). One of the few HCV vaccination strategies capable of inducing strong, broad, and multispecific T cell responses against the NS proteins of HCV (NS3-NS5) is the adenovirus prime and DNA boost vaccination regimen, which was evaluated in the chimpanzee model, and, relatively high frequencies of CD4+ (0.5%) and CD8+ (1.0–1.5%) T cells directed against NS3-NS5 were found after the last vaccination (21). All five animals had significantly reduced HCV RNA levels after a heterologous virus challenge, and the four chimpanzees with the most robust T cell responses resolved the infection. Although three of five chimpanzees in the control group also managed to clear the HCV infection, the results of this study underscore the importance of a strong, broad, and multispecific cellular immune response against HCV for viral control.

Among the NS proteins, cellular responses against the NS3 protein in particular have been shown to be associated with viral clearance (22, 23). In addition, the NS3 protein is one of the more conserved HCV Ags among the HCV genotypes. Because HCV is a highly mutable RNA virus with overall intergenotype variations of ~30% at the nucleotide and deduced amino acid level (24), it might be advantageous to apply NS3-based vaccines to obtain broad protection. Adenoviruses as vaccine vectors have been shown to be potent inducers of CD8+ T cell responses (25–27). Recently our laboratory found that linkage of the vaccine Ag to the murine invariant chain (II) can increase Ag presentation on MHC class I molecules resulting in enhanced, broadened, and prolonged CD8+ T cell responses (28). The modest results reported in the field of HCV vaccine imply that it is difficult to induce cellular immune responses to the NS proteins of HCV (17–21). We therefore hypothesized that an adenoviral-based vaccine encoding NS3 linked to II would improve the NS3-specific cellular responses.

In this study, the AdInNS3 vaccine was found to improve the CD8+ T cell response by increasing the frequency of NS3-specific CD8+ T cells, accelerating the cellular response, and inducing increased CD8+ T cell-mediated cytotoxicity compared with the AdNS3 vaccine. Furthermore, the AdInNS3-induced CD8+ memory T cells were of a high quality, because the majority were polyfunctional (IFN-γ, TNF-α, and IL-2) central memory T cells (Tcm) that expressed the phenotypic cell surface markers CD27 and CD127, which are associated with long-term survival of Ag-specific memory CD8+ T cells and maintenance of T cell memory. Protective immunity was evaluated in a vaccinia-virus murine challenge model.

**Materials and Methods**

**Mice**

Female C57BL/6 and BALB/c mice were obtained from Taconic Farms. C57BL/6.SJL mice, IFN-γ knockout (KO) mice, perforin KO mice, and IFN-γ-perforin double-KO mice were bred locally from breeder pairs originally obtained from The Jackson Laboratory. All mice used in this study were 7–10 wk old and housed in a specific pathogen-free facility. All experiments were approved by the local animal ethics council and were performed in accordance with the national experimental guidelines.

**Adenoviral vectors and vaccination**

The full-length of NS3 J4 (631 aa) (29), were amplified by PCR from plasmid pcV-J4L6S and cloned into the pacCMV shuttle plasmid with or without II. Homologous recombination of the pacCMV shuttle plasmid and Hu-Ad5 was performed in HEK 293 cells using standard methods (30). After purification, adenoviral stocks were aliquoted and frozen at ~80˚C in 10% glycerol stocks. The NS3 J4 insert was verified by sequencing and restriction enzyme digestion. Infectivity of the adenovirus stocks was determined with the Adeno-X rapid Titer kit. AdOVA and AdInP (nucleoprotein from lymphocytic choriomeningitis virus [LCMV]) control viruses were produced as described previously (28). Mice to be vaccinated were anesthetized and injected with 2 × 10^7 infectious units in 30 μl s.c. in the right hind footpad (31).

**Peptides used for stimulation**

A panel of 98 overlapping 11–15 mer peptides spanning the entire NS3 protein (strain J4L6S, genotype 1b) was obtained through the National Institutes of Health Bio Defense and Emerging Infections Research Resources Repository, NIAID, National Institutes of Health. In addition, Web-based CD8+ T-cell epitope prediction program netMHC3.0 (http://www.cbs.dtu.dk/services/NetMHC/) was used to predict NS3-specific epitope candidates, and selected peptides were obtained from Sigma-Aldrich. All peptides were used in vitro in concentrations of 2–4 μg/ml.

**Cell preparation**

Splenoocytes from mice were removed aseptically and transferred to HBSS. Single-cell suspensions were obtained by pressing the spleen through a fine steel mesh (70 μm), followed by centrifugation and two washes in HBSS before resuspension in RPMI 1640 cell culture medium containing 10% FCS supplemented with 2-mercaptoethanol, and penicillin-streptomycin.

**Flow cytometry**

After vaccination of mice with AdInNS3 or AdNS3, the frequencies of epitope-specific CD8+ T cells were determined by intracellular cytokine staining after 6 h of incubation with relevant peptides at 37˚C, 5% CO2 (1 h without and 5 h with monensin [3 μM]). After incubation the cells were stained with Abs for cell surface markers (PerCP-Cy5.5-CD8, allophycocyanin-Cy-CD4, and FITC-CD44) and Abs for intracellular cytokines (PE-CY7-IFN-γ, allophycocyanin–TNF-α, and PE-IL-2). Phenotypic characterization of the epitope-specific CD8+ T cells was performed by extracellular staining using the following Abs: PE-CD43 (activated glycoform, clone BiB11), FITC-CD27, and allophycocyanin-CD127. Samples were run on either FACSCalibur or LSRII (BD Biosciences) flow cytometers and analyzed using FlowJo software (Tree Star).

**Assessment of specific cell lysis in vivo**

C57BL/6 mice were vaccinated with AdInNS3 or AdNS3, and 14 or 60 d post-vaccination (p.v.) the mice were injected with peptide-pulsed, CFSE-labeled target cells from either C57BL/6 (CD45.2) or C57BL/6.SJL (CD45.1) mice. Single-cell suspensions of target cells were obtained by pressing spleens through a fine steel mesh (70 μm) followed by wash and resuspension in HBSS. C57BL/6 splenocytes were pulsed with relevant peptide (NS370–77 or NS3500–510), and C57BL/6.SJL splenocytes were pulsed with irrelevant peptide (LCMV Gp34.1; 30 min at 37˚C). After peptide pulse the cells were counted and adjusted to 1 × 10^7 cells/ml. The cells pulsed with NS370–77 peptide were labeled with CFSElow (0.2 μM) and mixed 1:1 with C57BL6.SJL cells pulsed with irrelevant peptide and were labeled with CFSEhigh (2 μM). The cells pulsed with NS3500–510 peptide were labeled with CFSElow (0.2 μM) and mixed 1:1 with C57BL6.SJL cells pulsed with irrelevant peptide and were labeled with CFSEhigh (2 μM). CFSE labeling was performed at 37˚C for 30 min. FBS (1/10 of total volume) was added to stop the reaction, and the cells was placed on ice for 2 min and washed three times in HBSS. Cell number was adjusted to 3.3 × 10^7 cells/ml, and 300 μl of the cell suspension was injected i.v. into the recipient mouse. After 5 or 8 h, the mice were sacrificed and splenocytes were isolated and labeled with PerCP-Cy5.5-CD45.2 and fixed in 1% paraformaldehyde. Specific cell lysis was determined by flow cytometry for the two epitopes NS370–77 and NS3500–510 (defined as the CFSElow population, respectively) and calculated using the equation (1 – [No. of CD45.2-positive cells + No. of CD45.2-negative cells]/100), as described previously (32).

BALB/c mice were vaccinated as described 60 d prior to the assay. Because no allotypic marker was available in these mice, the mice were injected with peptide-pulsed CFSE or CellTrace Violet (Invitrogen)–labeled targets cells from naive BALB/c mice. Cells pulsed with the relevant peptide (NS3514–531) were labeled with 5 μM CellTrace Violet, and cells pulsed with an irrelevant peptide (LCMV GP34.1) were labeled with 0.2 μM CFSE. After labeling the cells were counted, mixed 1:1, and adjusted to 3.3 × 10^7 cells/ml, and 300 μl of the cell suspension was injected i.v. into the recipient mouse. Naïve recipients served as controls. Eight hours after cell transfer the mice were sacrificed, and the splenocytes were isolated and analyzed by flow cytometry. Specific cell lysis is calculated as (1 –...
In vivo protection against infection with recombinant vaccinia virus expressing NS3

Recombinant HCV NS3 vaccinia viruses (33) were provided by C. Eisenbach. The three vaccinia viruses—named vAFL158, vAFL164, and vAFL464—contained different HCV 1b NS3 sequences. Mice were vaccinated with 2 × 10⁶ infectious units of AdIiNS3, AdNS3, or AdOVA; 14 d.p.v. the mice were challenged i.p. with 2 × 10⁶ PFU vAFL158, vAFL164, or vAFL464 recombinant NS3 vaccinia viruses. Five days later, mice were sacrificed and the ovaries were homogenized in 1 ml PBS and frozen at −80°C. Virus titres were determined by plating a 10% w/v solution in 10-fold dilutions on CV-1 monolayers in six-well plates and stained with 0.1% crystal violet after 48 h.

Statistical evaluation

Data are presented as mean ± SEM; n = 4–5. A nonparametric Mann–Whitney U test was used to compare quantitative data, unless noted otherwise; *p < 0.05, **p < 0.01, and ***p < 0.001. GraphPad Prism version 5 was used for statistical analysis.

Results

Identification of H-2d and H-2b restricted CD8+ T cell epitopes in NS3 HCV strain J4

With the use of the Web-based CD8+ T cell epitope prediction program netMHC3.0, J4 NS3-specific peptides with H-2d or H-2b binding motifs were predicted. In addition, a panel of 98 overlapping peptides spanning the NS3 sequence of strain J4 was obtained from National Institutes of Health Bio Defense and Emerging Infections Research Resources Repository, NIAID, National Institutes of Health. To easily identify peptides containing CD8+ T cell epitopes, a matrix was designed by dividing the peptides into 20 pools, each containing 10 peptides without overlapping sequences; each peptide was represented in two different pools. C57BL/6 and BALB/c mice were vaccinated with AdIiNS3 or an irrelevant vaccine (AdIiNP LCMV). On day 11 p.v., mice were sacrificed and the splenocytes were isolated and stimulated ex vivo for 6 h with the peptide pools or the predicted peptides. After identifying peptide pools containing potential epitopes, the matrix was applied to select the individual peptides tested in a secondary vaccination experiment. The identified CD8+ T cell epitopes are listed in Table 1. Two epitopes restricted to H-2d (C57BL/6 mice) and three epitopes restricted to H-2b (BALB/c mice) were found (Fig. 1). In C57BL/6 mice (Fig. 1A), the dominating epitope was NS3500–510 with ~3% CD44+IFN-γ producing CD8+ T cells among the entire CD8+ T cell population. In BALB/c mice (Fig. 1B) the major epitope was NS3514–531, with ~8.0% CD44+IFN-γ producing CD8+ T cells among all CD8+ T cells.

Linkage of HCV NS3 to MHC class II chaperone protein invariant chain markedly accelerated and enhanced the CD8+ T cell responses

Next we investigated whether the linkage of HCV NS3 to Ii could increase the frequencies of epitope specific CD8+ T cells. Mice were vaccinated with either AdIiNS3 or AdIiNS3, and the induced immune response was measured on days 7, 11, 14, and 22 p.v. to identify the peak T cell responses. Mice vaccinated with AdIiNS3 demonstrated enhanced and accelerated CD8+ T cell responses compared with mice vaccinated with AdNS3; this was seen for C57BL/6 and BALB/c mice (Fig. 2A). Regarding C57BL/6 mice, the AdIiNS3-induced CD8+ T cell response reached frequencies of 3.5%, which was 10-fold higher than the AdNS3-induced CD8+ T cell response (Fig. 2A). In BALB/c mice, 10.5% of the CD8+ T cells were CD44+IFN-γ in AdIiNS3-vaccinated mice at the peak of the response (day 14 p.v.), compared with only 4.5% in AdNS3-vaccinated mice (Fig. 2A). Furthermore, the linkage of HCV NS3 to Ii also appeared to broaden the vaccine-induced CD8+ T cell responses (Fig. 2B). The kinetics of the CD8+ T cell response against the minor epitopes was also analyzed, and a kinetics pattern similar to that found for the major epitopes was revealed (data not shown).

AdIiNS3 vaccination induced a sustained CD8+ T cell response with a poly-functional phenotype

The longevity of a vaccine-induced T cell response is an important quality, because long-term protection is an absolute requirement for prophylactic vaccination. The NS3-specific CD8+ T cells were therefore followed for up to 6 mo p.v. to determine the cytokine profile of the epitope-specific T cells during the effector and memory phase after a single vaccination. Groups of BALB/c and C57BL/6 mice were vaccinated with AdNS3 or AdIiNS3. The mice were sacrificed on day 14 or 180 p.v., and IFN-γ, TNF-α, and IL-2 production were measured by intracellular cytokine staining after a 6-h ex vivo stimulation with relevant peptides (NS3514–531 or NS370–77, Figs. 3, 4).

The analysis of BALB/c mice revealed that, at the early time point, NS3 linkage to Ii significantly enhanced the frequency of the CD8+ IFN-γ-producing T cells (6.7 ± 1.2% in the AdIiNS3 group compared with 2.1 ± 1.4% in the AdNS3 group). When evaluating the frequency of TNF-α–producing CD8+ T cells, the
frequency was also increased in AdIiNS3-vaccinated mice compared with the AdNS3-vaccinated mice with a similar fold increase, as seen for IFN-γ. IL-2 production was low in both vaccine groups, and no significant difference was observed (Fig. 3A, 3B). In the memory phase (day 180), the epitope-specific IFN-γ response was undetectable in mice receiving the AdNS3 vaccine, whereas mice from the AdIiNS3 vaccine group had an IFN-γ response of 2.4 ± 0.6%. (Fig. 3A, 3B).

A similar pattern was seen for the C57BL/6 mice (Fig. 4). At the early time point (day 14 p.v.), the frequency of CD8+ IFN-γ–producing T cells was significantly higher in the AdIiNS3 vaccine group compared with the AdNS3 vaccine group with 2.8 ± 0.4% compared with 0.7 ± 0.7%, respectively. The same pattern was seen for the frequency of TNF-α–producing CD8+ T cells in the two vaccination groups (Fig. 4A, 4B). At day 180 p.v., no CD8+ IFN-γ–producing T cells were detectable in mice vaccinated with AdNS3, whereas in the AdIiNS3 vaccinated mice the frequency of CD8+ IFN-γ–producing T cells was 0.4 ± 0.2% (Fig. 4A, 4B), which is significantly above the background. TNF-α and IL-2 production were low in both vaccination groups, and no significant difference between AdIiNS3- and AdNS3-vaccinated mice was seen. Thus, Ii linkage to NS3 expressed in an adenoviral vector increased the duration of the vaccine-induced immune responses.

Long-term protection against viral infection has been found to correlate with the presence of CD8+ T cells that are capable of co-producing several effector cytokines, in particular IFN-γ, TNF-α, and IL-2 (13–15). The presence of these multifunctional T cells is therefore a useful marker of vaccine-induced protection. Analysis of the distribution of IFN-γ single producers, IFN-γ and TNF-α double producers, and IFN-γ, TNF-α, and IL-2 triple producers during the effector phase and memory phase were performed for both mouse strains. The vaccine-induced NS3-specific effector T cells in BALB/c mice predominantly coproduced IFN-γ and TNF-α (66%), whereas one third of the NS3 specific CD8+ T cells were IFN-γ single producers and a small fraction of cells were triple-cytokine producers (4%). In the memory phase, two thirds of the epitope-specific CD8+ T cells produced all three cytokines (61%) and one third coproduced IFN-γ and TNF-α (32%; Fig. 3C). For the vaccinated C57BL/6 mice, it was only possible to perform this analysis on day 14 p.v. as the T cell frequency on day 180 p.v. was too low for any meaningful subdivision of the virus-specific CD8+ T cells. Forty-four percent of the NS370–77–specific effector CD8+ T cells coproduced IFN-γ and TNF-α; 47% of the CD8+ T cells were single IFN-γ producers, and 9% produced all three cytokines (Fig. 4C).

**Linkage of NS3 to Ii induces high-quality memory CD8+ T cells**

To further characterize the AdIiNS3-induced memory CD8+ T cells, we found it pertinent to analyze these cells in regard to changes in their surface phenotype as a function of time. Currently, it is generally accepted that functionally good memory CD8+ T cells express high levels of CD127 followed the pattern seen for CD27 as the expression of CD127 followed the pattern seen for CD27 as the cytokine increased. The presence of these multifunctional T cells is therefore a useful marker of vaccine-induced protection. Analysis of the distribution of IFN-γ single producers, IFN-γ and TNF-α double producers, and IFN-γ, TNF-α, and IL-2 triple producers during the effector phase and memory phase were performed for both mouse strains. The vaccine-induced NS3-specific effector T cells in BALB/c mice predominantly coproduced IFN-γ and TNF-α (66%), whereas one third of the NS3 specific CD8+ T cells were IFN-γ single producers and a small fraction of cells were triple-cytokine producers (4%). In the memory phase, two thirds of the epitope-specific CD8+ T cells produced all three cytokines (61%) and one third coproduced IFN-γ and TNF-α (32%; Fig. 3C). For the vaccinated C57BL/6 mice, it was only possible to perform this analysis on day 14 p.v. as the T cell frequency on day 180 p.v. was too low for any meaningful subdivision of the virus-specific CD8+ T cells. Forty-four percent of the NS370–77–specific effector CD8+ T cells coproduced IFN-γ and TNF-α; 47% of the CD8+ T cells were single IFN-γ producers, and 9% produced all three cytokines (Fig. 4C).

**AdIiNS3 induced epitope specific CD8+ T cells with increased cytotoxicity**

Next we investigated the cytotoxic activity of the vaccine-induced T cells. Splenocytes from naive C57BL/6 (CD45.2) mice were labeled with CFSE and pulsed with either NS370–77 (CFSEhigh) or NS3500–510 (CFSElow), and splenocytes from C57BL/6 SJL (CD45.1) mice were pulsed with irrelevant peptide. The cells...
were mixed 1:1 and adoptively transferred into C57BL/6 mice vaccinated with AdNS3 or AdIiNS3 14 or 60 d earlier. Five to 8 h after cell transfer, the mice were sacrificed and specific cell lysis was determined by flow cytometry. At day 14 p.v., we found 76.7 ± 6.13% specific killing of the NS3 70–77-loaded target cells in the AdIiNS3 vaccine group compared with 18.3 ± 7.2% specific killing of the NS3 70–77-loaded target cells in the AdNS3 vaccine group (Fig. 6A). For the subdominant epitope, we found 49.4 ± 16.8% specific killing of the NS3 500–510-loaded target cells in the AdIiNS3 group compared with 12.1 ± 4% specific killing of the NS3 500–510-loaded target cells in the AdNS3 vaccine group (Fig. 6A). On day 60 p.v., we found 19.3 ± 4.6% specific killing of the NS3 70–77-loaded target cells in the AdIiNS3-vaccinated animals compared with 0.69 ± 2.7% in the AdNS3-vaccinated animals (Fig. 6B). For the subdominant epitope we found 4.6 ± 1.8% specific killing of the NS3 500–510-loaded target cells in the AdIiNS3-vaccinated animals compared with 0.64 ± 1.8% specific killing of the NS3 500–510-loaded target cells in the AdNS3-vaccinated animals (Fig. 6B). In the memory phase (day 60), the cytotoxic activity of the AdNS3-induced CD8+ T cells was below the detection limit, whereas the cytotoxic potential of the AdIiNS3-induced CD8+ T cells was still readily detectable (Fig. 6B). Thus, AdIiNS3-induced effector T cells with increased cytotoxic activity compared with the AdNS3-induced T cells.

To ascertain that AdIiNS3 vaccination also induced superior effector T cell activity in BALB/c mice, we analyzed the in vivo cytotoxic activity against NS3514–531-loaded target cells in these mice on day 60 p.v. In the absence of an allotypic marker in BALB/c mice, Celltracel Violet and CFSE were used to label cells loaded with NS3514–531 or a control peptide, respectively. Similar to the situation in C57BL/6 mice, AdIiNS3-vaccinated BALB/c mice displayed markedly higher Ag-specific killing activity (46.6 ± 9.3%) than did BALB/c mice vaccinated using AdNS3 (14.34 ± 12.1%; Fig. 7). Thus, AdIiNS3 vaccination clearly induced a better vaccine-specific cytotoxic response than did AdNS3 vaccination, and this difference persisted for several months regardless of the mouse strain tested.

In vivo protection against infection with recombinant vaccinia virus expressing HCV NS3

To test the potential of the AdIiNS3 vaccine-induced immune response to combat a live virus challenge, three different recombinant vaccinia viruses expressing NS3 from HCV genotype 1b was used (33). The three recombinant NS3 vaccinia viruses differed by ∼5–9% in the NS3 aa sequence compared with the vaccine-encoded NS3. C57BL/6 mice were vaccinated with AdNS3, AdIiNS3, or AdOVA (negative control), and 14 d p.v. the animals were challenged with 2 × 10⁶ PFU of the NS3 vaccinia viruses i.p. Five days later, the ovaries were extracted and the vaccinia virus titers were determined (Fig. 8). Compared with the lowest level of infection detected in directly matched control, vaccinated mice, 11 of 15 and 13 of 15 mice vaccinated with AdNS3 and AdIiNS3 presented with reduced ovarian virus titers and, notably, in all three challenge experiments the AdIiNS3-vaccinated mice had a reduced average viral load compared with AdNS3-vaccinated mice. In the experiment with vAF164 and vAF464, the AdNS3- and AdIiNS3-vaccinated mice had significantly lower viral load compared with their directly matched
controls (Fig. 8B, 8C), whereas in the challenge experiment using vAF158 (Fig. 8A), two of five mice had cleared the vaccinia virus infection in the AdIiNS3 group on day 5 compared with no virus clearance in the AdNS3 group.

Two of the vaccinia viruses (vAF158 and vAF464) had mutations located in at least one of the two identified CD8+ T cell epitopes compared with the NS3 sequence encoded by the adenovirus-based vaccines (Table II). To evaluate the importance of these differences, we tested two peptides with altered sequences; a third peptide could not be synthesized to sufficient quality. C57BL/6 mice were vaccinated with AdNS3 and AdIiNS3, and at day 11 p.v. splenocytes were isolated and stimulated with peptides identical to the epitopes encoded by the vaccine or with the variant NS3500–510 (VTQMYTNV) and NS3300–510 (WYELTPAETV) peptides. The altered sequence in the NS350–77 epitope did not alter the cytokine response (Fig. 8D), whereas the mutation in the NS3300–510 significantly reduced the cytokine response (Fig. 8E).

**IFN-γ production is required for recombinant NS3 vaccinia virus clearance**

To define the mechanism by which the vaccinia virus challenge is eliminated, we performed NS3 vaccinia virus challenge experiments with IFN-γ KO mice, perforin KO mice, and IFN-γ–perforin double-KO mice in addition to wild type C57BL/6 mice. Mice were vaccinated with AdIiNS3 or AdOVA (as negative control). At day 14 p.v., the mice were challenged with 2 × 10^6 PFU i.p. of the vAF164 recombinant NS3 vaccinia virus (Fig. 9). A significant difference was seen between the AdIiNS3-vaccinated C57BL/6 mice and the AdIiNS3-vaccinated IFN-γ KO mice. Mice were vaccinated with AdIiNS3 or AdOVA (as negative control). At day 14 p.v., the mice were challenged with 2 × 10^6 PFU i.p. of the vAF164 recombinant NS3 vaccinia virus (Fig. 9). A significant difference was seen between the AdIiNS3-vaccinated C57BL/6 mice and the AdIiNS3-vaccinated IFN-γ KO mice. Similarly, a significant difference was found between the AdIiNS3-vaccinated C57BL/6 mice and the double IFN-γ–perforin KO mice. No difference was seen between the wild type mice and the perforin KO mice. These results suggest that the control of infection with recombinant HCV vaccinia virus depends predominantly on IFN-γ release and to a lesser extent on direct cytolytic activity mediated via perforin.

**Discussion**

In this study, we have shown that the technology of linking an HCV Ag to an MHC class II chaperone protein invariant chain has a dramatic effect on the vaccine-induced CD8+ T cells. We found that linkage of Ii to HCV NS3 enhanced, accelerated, and broadened the CD8+ T cell response in a similar manner as seen in the LCMV model (28, 40). The Ad-IiNS3–induced CD8+ T cell frequencies shown in this study are, to our knowledge, considerably higher than any of the previously published frequencies obtained after vaccination with HCV NS proteins (17–20). Moreover, these high CD8+ T cell frequencies were obtained after a single vaccination compared with other vaccination studies in which multiple heterologous prime boost vaccinations were required to reach even lower levels (20, 21).

The findings in previous vaccination studies, together with the data presented in this study, support a conclusion according to which the NS3 Ag is intrinsically poorly immunogenic and vaccine-induced responses strongly benefit from technologies that enhance Ag presentation. Improvement of the immunogenicity, by Ii linkage to Ag or other related technologies, might primarily be an advantage for Ags against which it is inherently difficult to mount an immune response, such as HCV NS3. In support of this, another recent study modified an adenoviral vector by fusing the Ag of interest to glycoprotein D (a molecule blocking the HVEM-BTLA negative regulation of B and T cell activation) (41). They found that this modification increased the immunogenicity by 70-fold for the HPV E7E6E5 Ag, but only a minor increase of 1.5-fold was seen for the HIV-1 gag Ag, which on its own is highly immunogenic (41). The fact that Ii linkage to HCV NS3 improved the CD8+ T cell response in two different mouse strains with different MHC haplotypes (Fig. 2) implies that the beneficial effect of Ii
linkage to NS3 in regard to the immunogenicity of the vaccine might be a general phenomenon and therefore also an advantage in other animal species.

Memory CD8+ T cells capable of producing several cytokines (IFN-γ, TNF-α, and IL-2) simultaneously are primarily of the central memory phenotype (14, 42). T CM cells have the capacity to produce relatively high amounts of IL-2 and have a high proliferative potential upon secondary challenge (34). When we analyzed the distribution of polyfunctional T cells in the AdIiNS3-induced memory population, we found that the majority of the Ag-specific CD8+ T cells were triple-cytokine producers (Fig. 3C). Because polyfunctional T CM-like cells are found to be important for viral control in other chronic viral infections such as HIV and LCMV (13–15), the AdIiNS3-induced memory T cells observed in this study are considered to be of high quality and have a desired T cell phenotype. In addition, expression of the phenotypical markers CD27, CD43 (the activated glycoform), and CD127 (IL-7Rα) can be used to characterize vaccine-induced memory T cells and their recall potential (35–37, 43). Thus, it is generally accepted that functionally good memory CD8+ T cells express high levels of CD127 (IL-7Rα required for Ag-independent survival) and CD27 (a marker of long-term survival), whereas the expression of CD43 (activated isoform) is low in these cells (34–39). It is pertinent, therefore, that ~60% of vaccine-induced NS3514–531 epitope-specific memory CD8+ T cells expressed the cell surface marker CD127, which is associated with long-term maintenance of T cell memory (36) and correlates with a successful virologic control (data not shown).

**FIGURE 5.** Phenotypic analyses of the NS3-specific CD8+ T cells in BALB/c mice 14 and 180 d p.v. BALB/c mice were vaccinated with AdIiNS3, and at days 14 or 180 p.v. splenocytes were isolated and stimulated with NS3514–531 for 6 h, followed by staining with Abs for the surface markers CD8, CD43, CD27, and CD127 (IL-7Rα) and intracellular staining for IFN-γ. Epitope-specific CD8+ T cells were identified as IFN-γ-positive T cells. A, Day 14. B, Day 180. The numbers in each quadrant indicate the percentage of gated cells, and the dot plot is representative of five individual mice.

**FIGURE 6.** Cytotoxic potential of the AdIiNS3 and AdNS3 vaccine-induced CD8+ T cells in C57BL/6 mice. C57BL/6 mice were vaccinated with either AdNS3 or AdIiNS3. The specific cell lysis of NS370–77- and NS3500–510-loaded cells was determined in an in vivo cytotoxicity assay using CFSE-labeled target cells. A, Assay performed day 14 p.v. B, Assay performed day 60 p.v. Mice were sacrificed 5 h after cell transfer in the experiment performed at day 14, and 8 h post cell transfer in the experiment performed at day 60. Plots are representative of two independent experiments. Data represent mean ± SEM; n = 5. *p < 0.05, **p < 0.01.

**FIGURE 7.** Cytotoxic potential of the AdIiNS3 and AdNS3 vaccine-induced CD8+ T cells in BALB/c mice. BALB/c mice were vaccinated with either AdNS3 or AdIiNS3. The specific cell lysis of NS3514–531 epitope-specific memory CD8+ T cells was determined in an in vivo cytotoxicity assay using CFSE and cell trace violet-labeled target cells. Assay was performed at day 60. Mice were sacrificed 8 h after cell transfer. Data represent mean ± SEM; n = 5. **p < 0.01.
outcome of HCV infection in humans (44). In addition, ~60% of the induced NS3-specific memory T cells expressed the cell surface marker CD27, a marker of long-lived memory T cells (35) (Fig. 4). In addition, the results shown in Fig. 4 suggest that the AdIiNS3 vaccine induces memory T cells with good recall potential, because the majority of the epitope-specific T cell population expressed low levels of CD43, a marker of recent activation, combined with high levels of CD27 expression. This combination of phenotypic markers (CD43low/CD27high) was recently found to be an indicator for a good recall potential upon a secondary challenge with Sendai virus (43) and permanent virus control in mice infected with LCMV, a virus with the potential to establish chronic infection (45). Moreover, a study comparing the response to different human viral infections including HCV suggests that Ag-experienced CD27⁺CD8⁺ cell are early differentiated memory cells (46).

Apart from the capacity for cytokine production, cytotoxic T cells are often important for efficient virus control. The cytotoxic activity in mice vaccinated with the two vaccines was tested in vivo, and it was observed consistently that Ad IiNS3 vaccination induced higher cytolytic activity compared with AdNS3. From the data shown in Figs. 6 and 7, it is not possible to determine whether this difference is due to more efficient cytotoxic CD8⁺ T cells in the AdIiNS3 group compared with the AdNS3 group or due to the increased frequency of NS3-specific T cells induced in the AdIiNS3 group compared with the AdNS3 group. However, the latter is almost certainly a contributing factor, as judged from the numbers of cytokine-producing cells revealed in these mice. The presence of cytotoxic, NS3-specific CD8⁺ T cells at day 60 p.v. (Figs. 6, 7) in AdIiNS3–vaccinated mice of both mouse strains confirms that NS3-specific memory T cells were generated and have the ability to execute relevant effector functions, even in C57BL/6 mice in which it was difficult to analyze these cells ex vivo during the memory phase.

To further characterize the functional capacities of the vaccine-induced CD8⁺ T cells, mice were challenged with vaccinia virus encoding heterologous NS3 (genotype 1b). The results from the vaccinia virus challenge studies suggest that both vaccines have the potential to induce some antiviral protection, but overall the AdIiNS3 vaccine seemed to produce more consistent results. This trend fits well with our finding that clearance of the challenge infection is mediated by IFN-γ production (Figs. 8, 9) and that the AdIiNS3 vaccine consistently induced higher responses in regard to this parameter. The requirement for IFN-γ in the control of vaccinia virus infection in mice is not a new observation and is

Table II. Amino acid differences in the recombinant NS3 vaccinia virus compared with NS3 HCV J4 vaccine

<table>
<thead>
<tr>
<th>NS3 Vaccinia Virus or Vaccine</th>
<th>NS330-77</th>
<th>NS3300-510</th>
<th>Mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>vAF158 VTQMYTNV</td>
<td>WYELTPAESV</td>
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<tr>
<td>vAF164 TIFQMYTNV</td>
<td>WYELTPAESV</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>vAF464 VIQMYTNV</td>
<td>WYELTPAESV</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>AdIiNS3 vaccine</td>
<td>ITQMYTNV</td>
<td>--</td>
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</tr>
</tbody>
</table>

Amino acid differences (underscored letters) between the NS3 sequence encoded by the vaccinia virus and the adenovirus vaccine in the two H-2b epitopes. The overall amino acid differences between the NS3 encoded by the vaccinia viruses and the NS3 encoded by the adenovirus vaccine is 4.75% for vAF158, 4.9% for vAF164, and 9.0% for vAF464.
consistent with published observations (47). The two CD8+ T cell epitopes identified in the C57BL/6 mice differed by 1 or 2 aa when comparing the vaccinia virus encoded and the adenovirus encoded NS3 sequences. To test the importance of this mismatch, the IFN-γ production was measured upon stimulation with the vaccine-encoded NS3 epitopes and the vaccinia virus-encoded NS3 epitopes. For the NS370–77 epitope, no effect of the altered amino acid sequence was seen (Fig. 8D), whereas for the NS3500–510 epitope the amino acid mutation had a deleterious effect on the IFN-γ response (Fig. 8E). Although the first observation may be explained readily by assuming that the amino acid at position 70 is outside of the critical interaction region involved in T cell recognition of the peptide–MHC complex, we have no obvious explanation regarding the second result. The anchor residues do not appear to be canonical, thus complicating the interpretation, but it is possible that the S-to-T mutation significantly affects TCR binding. Interestingly, when comparing these findings with the results from the vaccinia challenge experiment (Fig. 8A, 8B), no related difference in protection was observed. It is possible that a difference in IFN-γ response could have been revealed with careful titration of the epitope with the altered sequence. It is also recognized that the challenge model used in this study is based on vaccinia virus, whose replication strategy and ability to induce an immune response differ greatly from that of HCV. Furthermore, vaccinia virus, whose replication strategy and ability to induce an immune response differ greatly from that of HCV. Additionally, the NS3 Ag requires careful titration of the epitope with the altered sequence. It is also possible that the S-to-T mutation significantly affects TCR binding. Consequently, when comparing these findings with the results from the vaccinia challenge experiment (Fig. 8A, 8B), no related difference in protection was observed. It is possible that a difference in IFN-γ response could have been revealed with careful titration of the epitope with the altered sequence. It is also recognized that the challenge model used in this study is based on vaccinia virus, whose replication strategy and ability to induce an immune response differ greatly from that of HCV. Furthermore, the antiviral protective capacities of specific CD8+ T cell subsets are closely related to the nature of the challenging pathogen, and different viral infections may require different T cell functions for efficient clearance (48). Because no small animal HCV challenge model exists, the use of the NS3 vaccinia virus challenge model was found useful to evaluate the antiviral response of the vaccine-induced T cells.

In conclusion, our laboratory has shown previously that Ii-linkage of the vaccine Ag could enhance, broaden, accelerate, and prolong CD4+ and CD8+ T cell responses toward the model virus LCMV independent of vaccination platform (28, 40). The current study further explored the benefits of the Ii linkage technology by applying it to an Ag of human disease relevance, HCV NS3, which moreover is well known for its low immunogenicity (17–20). In this study, we show that the intrinsically poorly immunogenic Ag HCV NS3 benefits from the Ii technology mediating increased Ag presentation, and that this vaccination strategy led to responses markedly higher than what has previously been reported. Moreover, the induced CD8+ T cell responses were of a high quality and associated with the potential to control viral infection. Thus, this vaccine seems to represent a relevant new approach for inducing a strong cell-mediated immune response to HCV. However, given that the most effective HCV vaccines probably will require the capacity to induce both humoral and cellular immunity, what are the perspectives for induction of relevant CD4 and B cell responses? Although not investigated in this report, it is extremely likely that also the antiviral CD4+ T cell response is improved considering our experiences from the LCMV model (28). Clearly this should improve the induction of CD8+ T cell memory (49) and potentially increase T cell help for the Ab response. However, for the latter to be biologically relevant, the inclusion of an HCV surface protein as target Ag for neutralizing Abs would be required. Whether more effective neutralizing Ab responses may be induced using Ii chain strategy, we do not know with certainty. Our experiences with induction of Abs to different viruses using this approach have varied (P.J. Holst, A.R. Thomsen, and J.P. Christensen, unpublished observations). The reason for this could be that although CD4+ T cell help may be improved, tethering of the Ag to Ii chain may impose some restrictions on the presentation of structural epitopes to the B cells. It is therefore likely that the final vaccine to HCV may have to encode a surface Ag (E1/E2) unlinked to Ii chain for a neutralizing Ab response in addition to a more conserved nonstructural protein (e.g., NS3) tethered to invariant chain for strong cell-mediated immunity. Nevertheless, because it is known that invariant chain is conserved structurally and functionally in mammalian, including humans and nonhuman primates, we find the present results sufficiently encouraging to propose this HCV vaccine as a candidate for evaluation in the chimpanzee model.

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Disclosures
The authors have no financial conflicts of interest.

References


