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A Synthetic Codon-Optimized Hepatitis C Virus Nonstructural 5A DNA Vaccine Primes Polyfunctional CD8+ T Cell Responses in Wild-Type and NS5A-Transgenic Mice

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The hepatitis C virus (HCV) nonstructural (NS) 5A protein has been shown to promote viral persistence by interfering with both innate and adaptive immunity. At the same time, the HCV NS5A protein has been suggested as a target for antiviral therapy. In this study, we performed a detailed characterization of HCV NS5A immunogenicity in wild-type (wt) and immune tolerant HCV NS5A-transgenic (Tg) C57BL/6J mice. We evaluated how efficiently HCV NS5A-based genetic vaccines could activate strong T cell responses. Truncated and full-length wt and synthetic codon-optimized NS5A genotype 1b genes were cloned into eukaryotic expression plasmids, and the immunogenicity was determined after i.m. immunization in combination with in vivo electroporation. The NS5A-based genetic vaccines primed high Ab levels, with IgG titers of >10^4 postimmunization. With respect to CD8+ T cell responses, the coNS5A gene primed more potent IFN-γ-producing and lytic cytotoxic T cells in wt mice compared with NS5A-Tg mice. In addition, high frequencies of NS5A-specific CD8+ T cells were found in wt mice after a single immunization. To test the functionality of the CTL responses, the ability to inhibit growth of NS5A-expressing tumor cells in vivo was analyzed after immunization. A single dose of coNS5A primed tumor-inhibiting responses in both wt and NS5A-Tg mice. Finally, immunization with the coNS5A gene primed polyfunctional NS5A-specific CD8+ T cell responses. Thus, the coNS5A gene is a promising therapeutic vaccine candidate for chronic HCV infections.  

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C hronic liver disease caused by the hepatitis C virus (HCV) is associated with development of fibrosis, cirrhosis and hepatocellular carcinoma. It is estimated that 170 million individuals are infected by HCV worldwide (1, 2). The HCV has a positive sense ssRNA genome of ∼9.6 kb, which encodes 10 structural and nonstructural (NS) proteins. The current standard-of-care therapy for HCV genotype 1 infection is based on a triple combination composed of pegylated IFN-α, ribavirin, and a direct-acting antiviral (DAA) compound. Numerous DAAs are in clinical development, and thus far, two NS3 protease inhibitors have reached the market. Data show an improved sustained virological response (SVR) in patients treated with NS3 protease inhibitors (3, 4). For HCV nongenotype 1 infections, the therapy is composed of pegylated IFN-α, ribavirin. Individuals that clear an acute HCV infection spontaneously or by standard-of-care treatment more commonly have CD4+ and CD8+ T cells to multiple HCV proteins, whereas those who progress to a chronic infection lack these responses. One explanation may be that these chronic carriers have dysfunctional T cells (5–9). Hence, one approach to reactivates these dysfunctional T cells is therapeutic vaccination (10–12). Until now, no vaccine is available for HCV, although several candidates have been evaluated in clinical phase I/II trials (11, 13–15). By using DNA-based vaccines, one may activate the preferred type of immunity, including both humoral and cellular immune responses, in small animals and humans. The HCV NS5A is of particular interest because it is recognized by cytotoxic T lymphocytes during natural infection and resolution of acute HCV in humans (16). Thus, we decided to evaluate HCV NS5A as a therapeutic DNA vaccine candidate for chronic HCV infection, because relatively few studies have investigated NS5A as a potential vaccine (17–19). The activation of a potent antiviral T cell response is a prerequisite for a functional therapeutic vaccine in the presence of the homologous virus. We therefore use NS5A-Tg mice in addition to wild-type (wt) mice, because they better represent a model of an infected host with impaired or dysfunctional immune response to NS5A (20). In addition, NS5A is an interesting target for DAAs, because clinical evaluation has shown promising results with improved SVRs (21–23). Importantly, these inhibitors seem effective across genotypes (24).

The function of NS5A is not completely understood, although it is known to be an essential component of the HCV replication complex (25, 26). The NS5A protein has no known enzymatic activity, and it is divided in three distinct structural domains, domain I, II, and III (27). Domain I is essential for RNA repli-
cation and has a zinc-binding motif, and the domain is a dimer as determined by crystallography (27). Domains II and III are less conserved and natively unfolded (28–30). Domain III is required for proper assembly of viral particles (31) but is not needed for RNA replication (32). NS5A has been described to interact both with cellular proteins like Raf-1, p53, PI3K, and Grb2, which are important for host cell signaling (20, 33), but also to interfere with components of innate immunity by inhibiting PKR and cyclophilin A (33–35). Hence, NS5A may affect IFN signaling pathways and production of proinflammatory cytokines. In addition, we have shown that NS5A interferes with adaptive immunity by protecting hepatocytes from cytolytic killing (20). In contrast, long-term intrahepatic NS5A protein expression does not induce spontaneous liver disease or cancer (20, 36). In this study, truncated forms of NS5A genes were investigated along with the full-length NS5A to define the immunogenicity of different domains of this viral protein.

The aim of this study was to develop and evaluate HCV NS5A genotype 1b DNA vectors as potential vaccine candidates for chronic HCV infection. We initiated a study to characterize the immunogenic properties of NS5A-vaccine candidates in wt and immunologically tolerant NS5A-Tg mouse models.

**Materials and Methods**

**Animals**

Inbred C57BL/6J mice were obtained from a commercial vendor (Charles River Laboratories, Sulzfeld, Germany). NS5A-Tg C57BL/6J mice were generated by backcrossing NS5A-Tg FVB/N mice (20) with C57BL/6J mice for >10 generations. Transgenic (Tg) mice were bred and maintained at Karolinska Institute (Division of Comparative Medicine, AKM), Clinical Research Centre, Karolinska University Hospital Huddinge (Stockholm, Sweden). Female mice were 6–16 wk of age at the start of the experiments. All experimental protocols involving animals were approved by the Ethical Committee for Animal Research at Karolinska Institutet.

The liver-specific expression of NS5A protein in C57BL/6J mice was under the control of the mouse albumin enhancer and promoter, followed by the rabbit β-globulin intron that enhances protein expression. A V5 tag and a polyadenylation signal from SV40 were inserted downstream of the NS5A gene.

All NS5A-Tg mice were genotyped by PCR to verify the transgene. Extraction of DNA from the tail was performed using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions, and the NS5A transgene and the reference gene tubulin were amplified by PCR.

**Peptides and proteins**

Peptides (87 synthesized 20-mer) corresponding to the complete NS5A protein sequence with 15 aa overlap were synthesized by automated peptide synthesis (37) (provided by ChronTech Pharma, Huddinge, Sweden). In addition, the SYFPEITHI database, version 1.0 (38) (http://www.syfpeithi.de), was used to predict H-2Db (9-mer) and H-2Kb (8-mer) epitopes. The following NS5A MHC class I binding peptides were used: NS5A 2251–2259 (VILDSFDPL, H-2Db) and 2252–2259 (ILDSFDPL, H-2Kb).

**Plasmids**

A wt NS5A genotype 1b gene [GenBank accession number D16435, http://www.ncbi.nlm.nih.gov/Genbank (39)] and eight truncated wtNS5A constructs were inserted into the pcDNA3.1 vector (Table I). A codon-optimized (co) NS5A genotype 1b gene was synthesized and inserted into the pVAX1 vector (Retrogen, San Diego, CA). The wtNS5A and coNS5A genes had 100% aa homology but differed by 23% in nucleotide sequence. The GC content was reduced from 60 to 58%.

The plasmid DNAs used for in vivo immunization were in competent *Escherichia coli* TOP10 cells and purified by using the Plasmid DNA Purification and QIAfilter Plasmid Mega Kit (Qiagen), according to the manufacturer’s instructions. The DNA was diluted in sterile PBS to 1 mg/ml, and the plasmid DNA concentration was determined spectrophotometrically (Biophotometer; Eppendorf, Hamburg, Germany). 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).

**Cell lines and primary cultures**

The RMA-S (TAP deficient) and EL-4 (T cell lymphoma) cell lines were cultured in RPMI 1640 medium supplemented with 5% FBS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA).

Primary cell cultures from spleen were cultured in complete CTL medium (RPMI 1640 medium supplemented with 10% FBS, 1 mM sodium pyruvate, 10 mM HEPES buffer, 1 mM nonessential amino acids, 50 µM 2-ME, 100 U/ml penicillin, and 100 µg/ml streptomycin [Invitrogen]). All cells were incubated in humidified incubators at 37°C with 5% CO2.

**Stable transfection of EL-4 cells**

The EL-4 suspension cells were stably transfected using TurboFect (Fermentas, Leon-Rot, Germany), according to manufacturer’s instructions. In brief, 5 × 10⁶ cells/well were seeded in a 24-well plate in 1 ml growth medium 24 h prior to transfection. Linearized DNA (1 µg) and 100 µl serum-free medium, mixed with 2 µl TurboFect, and incubated at room temperature for 20 min. The TurboFect/DNA mixture was then added dropwise to the cells without removing the growing medium. The cells were incubated for 48 h at 37°C and 5% CO2. After transfection, the EL-4 cells containing wtNS5A-pcDNA3.1 (aa 1–449) were grown in the presence of 800 µg/ml genetin (G418/ml [Invitrogen]) for 48 h and then singling diluted for single round of clones. This procedure was repeated twice. Expression of NS5A protein from the stably transfected EL-4 cells was analyzed by reverse transcription and PCR and Western blot.

**RNA extraction and RT-PCR**

Detection of NS5A expression in stably transfected NS5A-EL4 cells was performed by an RT-PCR to visualize NS5A mRNA. Total RNA was isolated using the TRIzol reagent, according to manufacturer’s instructions. In brief, 5 × 10⁷ cells/well were seeded in a 24-well plate in 1 ml medium 24 h prior to transfection. Linearized DNA (1 µg) was mixed with 100 µl serum-free medium, with 2 µl TurboFect, and incubated at room temperature for 20 min. The TurboFect/DNA mixture was then added dropwise to the cells without removing the growing medium. The cells were incubated for 48 h at 37°C and 5% CO2. After transfection, the EL-4 cells containing wtNS5A-pcDNA3.1 (aa 1–449) were grown in the presence of 800 µg/ml genetin (G418/ml [Invitrogen]) for 48 h and then singling diluted for single round of clones. This procedure was repeated twice. Expression of NS5A protein from the stably transfected EL-4 cells was analyzed by reverse transcription and PCR and Western blot.

**Immunizations**

Mice were immunized i.m. in the *tibialis cranialis* muscle with 50 µg plasmid DNA [coNS5A-pVAX1, wtNS5A-pcDNA3.1, or truncated wtNS5A constructs in pcDNA3.1 (Table I)] in a volume of 50 µl. Immediately following administration of the plasmid DNA, *tibialis cranialis* muscles were subsequently electroporated (EP) using the MedPulse DNA delivery system (DOS; Invivo Pharmaceuticals, Blue Bell, PA) (40). The mice were boosted at monthly intervals and were immunized one to three times. Protein-based immunizations were performed by a s.c. injection of 50 µg rNS5A protein (Mikrogen) diluted in PBS mixed with Freund’s (prime:...
complete, boost incomplete) adjuvant 1:1 to a final volume of 100 μl that was administered at the base of the tail.

In vivo challenge with tumor cells stably expressing NSSA

In vivo challenge with NSSA stable expressing EL-4 lymphoma cells was performed in naive and immunized mice 2 wk postimmunization using 1–1.5 × 10^6 tumor cells. The cells were washed, resuspended in 200 μl PBS, and inoculated s.c. in the right flank of the mouse. The kinetics of the tumor growth was determined by measuring the tumor volumes through the skin using a sliding caliper every second or third day, and the volume was calculated by using the formula: 0.5 × (tumor length × tumor diameter)^2 (41).

Detection of NSSA-specific Abs using ELISA

Serum from immunized C57BL/6J mice was collected at indicated time points via retro-orbital bleeding of Isofluran anesthetized mice. Detection of mouse IgG Abs to NSSA was performed as described previously (42). In brief, plates were coated with 1 μg/ml nNSSA (Mikrogen for protein- and GentScript for DNA-immunized mice). Mouse serum was added in serial dilution (starting dilution of 1:60 and then diluted 1:6). OD was read at 405 nm with a 620-nm background. The cutoff value was set to three times the OD value of a negative serum sample.

Sample preparation and Western blot analysis

Liver biopsies (100 mg) were homogenized using sonication (Vibra Cell Homogenizer; Chemical Instruments, Lidingö, Sweden) in 1 ml radioimmunoprecipitation assay buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.4], 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 1% SDS, 2 mM PMSE, 0.25 mM DTT, and 10 mM Na_2VO_4 (Sigma-Aldrich)). The liver homogenates were incubated on ice for 10–20 min and centrifuged at 12,000 × g for 2 min at 4°C, and the supernatant was transferred to a new vial and stored at −80°C until analysis. A total of 60 μg protein was separated on a SDS-PAGE 4–12% Bis-Tris gel. For running and blotting of protein gels, the XCell SureLock Mini-Cell system and iBlot equipment (Invitrogen) were used following the manufacturer’s protocol and as described previously (43). Abs targeted to rabbit anti-V5 (diluted 1:1,000) and goat anti-rabbit HRP (diluted 1:5000) were purchased from Sigma-Aldrich and DakoCytagomation (Glostrup, Denmark), respectively. Proteins were visualized using the ECL Plus Western blotting reagent (GE Healthcare, Uppsala, Sweden), according to the manufacturer’s protocol.

Detection of IFN-γ and IL-2–producing CTLs and Th cells by ELISPOT assay

Secretion of IFN-γ and IL-2 by splenocytes from pooled groups of mice was detected using a commercial ELISPOT assay according to manufacturers protocol (MacTech; Nacka Strand, Sweden) and as described previously (10). The number of spots was measured using the Aid ELISPOT reader system (version 2.6) with the ELISPOT 3.2.3 software (Autoimmum Diagnostika, Straussberg, Germany). Results are shown as the mean number of IFN-γ- or IL-2–producing spot-forming cells (SFCs) per 10^6 cells of triplicate values.

Detection of NSSA-specific CTLs by cytotoxicity assay (e.g., ^[51]Cr release assay)

Detection of NSSA-specific lytic cytotoxic T cells was performed using a ^[51]Cr release assay as described previously (44). In brief, splenocytes from cNSSA-pVAX1–immunized and nonimmunized mice were harvested and resuspended in complete CTL medium. The splenocytes were restimulated in vitro for 5 d in 25-ml flasks (e.g., T25) containing 25 × 10^6 spleen cells and 25 × 10^6 irradiated (2000 rad) syngenic splenocytes. The restimulation was performed in presence of the 9- or 8-mer peptides corresponding to the NSSA protein sequence. After 5 d of in vitro restimulation, a standard ^[51]Cr release assay was performed in 96-well V-bottom plates. The radioactivity was detected using a 1450 Microbeta Trilux scintillation counter, with the Microbeta Workstation Software 4.0 (PerkinElmer, Waltham, MA). The percentage of specific ^[51]Cr release was calculated according to the formula: ([experimental release – spontaneous release]/[maximum release – spontaneous release]) × 100. Maximum release was calculated from supernatants of cells that were lysed by addition of Triton X-100 (Sigma-Aldrich). Spontaneous release was determined from supernatants of cells incubated without effector cells. Results are shown as the mean percent-specific lysis of triplicate values.

Flow cytometry

Splenocytes from naive and vaccinated mice were stimulated overnight at 37°C (0.5 × 10^6 cells/well in 96 V-bottom plate) in complete CTL medium with 5 μg/ml NSSA_{2251-2259} (VILDSFDPL) peptide, 5 μg/ml NSSA_{2252-2259} (ILDSFDPL) peptide, 5 μg/ml OVA CTL peptide, PMA-ionomycin (100 ng/ml and 1 μg/ml, respectively), and medium control. After stimulation, the cells (0.5 × 10^6 cells/well in 96 V-bottom plate) were washed and resuspended in FACS buffer (PBS/1% FBS) and then incubated with Live and Dead (Invitrogen) in dark for 30 min on ice and blocked with Fc block (anti-mouse CD16/32; BD Biosciences, Franklin Lakes, NJ). Staining for cell surface markers with Abs against mouse CD107a-PE (1D4B) and CD8-PerCP (53-6.7) proceeded for 15 min on ice. The cells were then fixed with Cytofix/Cytoperm (BD Biosciences) for 20 min on ice, washed with permwash (BD Biosciences), and subsequently stained intracellularly with Abs against mouse CD3-Pacific Blue (17A2), IFNγ-FITC (XMG1.2), IL-2–allophycocyanin (JES6-5H4) and TNFα-Cy7 (MP6-XT22) in permwash for 30 min on ice. All Abs were purchased from BD Biosciences. Approximately 50,000 living lymphocytes were counted per sample with a Fortessa flow cytometer (BD Biosciences) and analyzed with the FlowJo software (Tree Star, Ashland, OR).

Splenocytes from individual naive and immunized mice were harvested and used directly ex vivo for NSSA-specific CD8+ T cell quantification. A total of 1 × 10^6 cells/well in 96 V-bottom plate were washed and resuspended in PBS/1% FBS (FACS buffer) and incubated 15 min in the dark at 20°C with R-PE-labeled H-2K^b Pro5 pentamer refolded with HCV NSSA_{2251-2259} (VILDSFDPL) or HBV core (MGLKFRQSL) from Prolimmune (Oxford, U.K.). After labeling, the cells were blocked with Fc block (anti-mouse CD16/32) for 15 min on ice, washed in FACS buffer, and stained with cell surface markers against mouse CD19-PE-Cy5 (clone 6D5) and CD8-FITC (clone KT15) (Prolimmune, Oxford, U.K.) for 20 min on ice. The cells were then fixed in 2% paraformaldehyde in PBS. A total of 150,000 events were subsequently acquired from each sample using a FACSCalibur flow cytometer (BD Biosciences) and analyzed using the CellQuest software. From a live lymphocyte gate, CD19^+ events were excluded, and the remaining cells were gated for CD8 expression. Frequency of NSSA H-2K^b (VILDSFDPL)-positive events within this population was determined.

RMA-S stabilization assay

The following peptides were evaluated for ability to stabilize the MHC class I molecule: NSSA_{2251-2259} (VILDSFDPL, ILS2252-2259) (ILDSFDPL), NSSA_3 (GAVQNEVTL), Hb:Ag CTL (MGLKFRQSL), and NSSA_{2248-2256} (NKVVILDSP) in five different concentrations (10, 5, 1, 0.5, and 0.1 μg/ml). RMA-S cells were incubated overnight at 2°C with different concentrations of NSSA and medium control (100 ng/ml and 1 μg/ml, respectively), and medium control. After incubation, the cells were washed and resuspended in FACS buffer and then stained in two separate tubes (1 × 10^6 cells for each peptide concentration) with Abs against mouse H-2D^b–FITC and H-2K^d–PE for 30 min on ice. A total of 30,000 events from each sample were counted on a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ) using the CellQuest software and analyzed using the FlowJo software (Tree Star, Ashland, OR).

Statistical analysis

All comparisons were performed using GraphPad InStat 3, Macintosh (version 3.0b, 2003; GraphPad Software, San Diego, CA) and Microsoft Excel 2008, Macintosh (version 12.2.8; Microsoft, Redmond, WA). The kinetic of measurements was compared using the area under the curve (Excel). Parametrical data were compared using the ANOVA (InStat 3), and nonparametrical data were compared with Mann–Whitney U test (InStat 3).

Results

Characterization of full-length and truncated NSSA expression constructs

To determine the immunogenic properties of the different domains of NSSA, we immunized mice with one wt full-length, one co full-length, and eight wt truncated NSSA constructs for their ability to activate NSSA-specific immune responses (Table I) (45). All constructs were verified by restriction enzyme digest, sequencing, and an in vitro transcription and translation assay for proper NSSA protein expression prior to immunization (data not shown).

Humoral immune responses after DNA or protein immunization with full-length and truncated NSSA expression constructs

To investigate the immunogenic properties of different NSSA-DNA constructs or protein, groups of C57BL/6 (H-2^b) mice were immunized with a single dose of 100 μg of the corresponding plasmids or proteins. Serum samples were collected on days 28 and 42 postimmunization, and the presence of NSSA-specific Abs was determined using ELISA.

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munized i.m. with 50 \( \mu \)g plasmid DNA in combination with in vivo EP using the MedPulser DDS apparatus (40) or s.c. with 50 \( \mu \)g rNS5A protein at the base of the tail. Analysis of anti-NS5A IgG titers after immunization revealed that DNA immunization induced an efficient Ab response, although it appeared later and had a lower magnitude than the NS5A protein immunization (Fig. 1A). NS5A IgG titers reached the plateau around week 6 after the first immunization (Fig. 1). Interestingly, immunization of mice with the different truncated and full-length DNA constructs showed that plasmids containing NS5A amino acid sequences corresponding to aa 327–449 were able to induce equally strong Ab responses as the full-length wt NS5A construct (Fig. 1E–I). In contrast, constructs containing amino acid sequences corresponding to aa 1–326 did not prime any detectable NS5A IgG Ab titers detectable in full-length NS5A (Fig. 1B–D). This suggests the humoral immune responses are mainly focused toward the C-terminal part of NS5A.

Cellular immune responses after DNA immunization with full-length NS5A expression constructs

To evaluate cellular immune responses primed by NS5A–DNA expression constructs we needed to identify NS5A MHC class I CTL epitopes in C57BL/6J (H-2\(^b\)) mice since there are no NS5A MHC class I epitopes identified in the H-2\(^b\) haplotype. A set of 87 synthetic 20-mer peptides (15 aa overlap) spanning the full-length NS5A gt1b gene was made for this purpose. In addition, MHC class I epitopes were predicted using the SYFPEITHI database version 1.0 (38) (http://www.syfpeithi.de). Screening for MHC class I NS5A epitopes was performed by harvesting spleens from NS5A–DNA-immunized wt C57BL/6J mice and restimulating cultures for 36 h in the presence of the 87 individual peptides in two concentrations (10 and 1 \( \mu \)g/ml) and thereafter determining IFN-\( \gamma \) production by ELISPOT assay. Identified and predicted peptides were tested for the stabilization of surface expression of MHC class I molecules on the TAP2–deficient RMA-S cell line (46, 47). Two NS5A peptides were identified that bound H-2\(^D\) and H-2\(^K\) molecules with high affinity. Further fine-mapping revealed two candidate peptides, one with high affinity for the H-2\(^D\) molecule (sequence VILDSFDPL, aa 2251–2259) and one for the H-2\(^K\) molecule (sequence ILDSFDPL, aa 2252–2259) (Fig. 2A, 2B). It was noted that these two epitopes share the same amino acid sequence but differ in their MHC restriction. To verify whether the newly identified peptides are processed and are immunogenic in vivo, we used two approaches to analyze NS5A epitope–specific immune responses. First, wt C57BL/6J mice were immunized with 50 \( \mu \)g coNS5A-pVAX1 i.m. in combination with EP. Second, NS5A-Tg mice (20) backcrossed to a C57BL/6J (H-2\(^b\)) background were immunized with the same dose of vaccine. The NS5A-Tg C57BL/6J mouse strain is a newly generated mouse lineage with constitutive hepatic NS5A protein expression (Fig. 2C) (20). Two weeks post-immunization, mice were sacrificed, and splenocytes were restimulated with the two peptides and analyzed for IFN-\( \gamma \) and IL-2 production by ELISPOT assay and for lytic activity by a \([\text{\[^{51}Cr]\]}\)
FIGURE 2. Characterization of NS5A-specific cellular immune responses in wt and NS5A-Tg mice. A peptide stabilization assay was used to identify epitopes binding to H-2Db (A) and H-2Kb (B). Results are presented as the lowest binding peptide concentration in μg/ml. Peptides below the detection limit are marked with ♦. (C) Detection of NS5A protein in NS5A-Tg C57BL/6J mice (lanes 1, 2), and a non-Tg wt C57BL/6J mouse (lane 3). Size marker (M), Magic Mark (Invitrogen). Quantification of NS5A-specific IFN-γ- and IL-2–producing T cells per 10^6 splenocytes determined by an ELISPOT assay. Wt C57BL/6J mice (D, G), NS5A-Tg (E, H), and nonimmunized wt C57BL/6J mice (F, I) (n = 5/group) were immunized i.m. once with 50 μg coNS5A-pVAX1, followed by in vivo electroporation. Data have been given as SFCs/10^6 splenocytes and SD. Cutoff has been set as 50 SFCs/10^6 splenocytes. NS5A-specific lytic CTL activity was determined by [51Cr] release assay in wt C57BL/6J (J, M), NS5A-Tg (K, N), and nonimmunized wt C57BL/6J (L, O) mice. Groups of five mice were i.m. immunized once with 50 μg coNS5A-pVAX1 or left untreated. Splenocytes from individual mice were restimulated in presence of NS5A peptides (NS5A2251–2259 or NS5A2252–2259), and lytic activity was determined by using peptide-loaded RMA-S cells. Values are presented as percent NS5A peptide–specific lysis. Values are given for E:T ratio of 60:1, 20:1, and 7:1. The presence of a statistical (Figure legend continues)
release assay using peptide-loaded RMA-S cells (Fig. 2D–O). These results revealed that both MHC class I NS5A peptides were processed and properly presented in vivo to induce T cell responses that were able to produce high levels of IFN-γ and IL-2 in wt mice (Fig. 2D, 2G). Interestingly, IFN-γ production was also detected in immunized mice with constitutive liver-specific NS5A protein expression, although at a lower magnitude (Fig. 2E, 2H). The NS5A-specific IFN-γ and IL-2 production was statistically significantly higher in immunized wt compared with NS5A-Tg mice (p < 0.001 and p < 0.01, respectively). This indicates that the NS5A-Tg mice have a partially impaired T cell response to NS5A. Moreover, we were able to detect low levels of IFN-γ production by splenocytes stimulated with rNS5A in wt mice but none in NS5A-Tg mice (Fig. 2D–F). No IL-2 production was detected from splenocytes stimulated with rNS5A (Fig. 2G–I). Moreover, the cytotoxic potential of the NS5A-specific T cells could be demonstrated in a [31]Cr release assay on peptide-loaded RMA-S target cells. Functional testing revealed that both peptides presented on MHC class I can be recognized and killed by in vivo primed NS5A-specific CTLs (Fig. 2J–O). There was no statistical significant difference between the lytic activity of the two peptides and between wt and NS5A-Tg mice, although the percent-specific lysis of target cells was somewhat lower in the NS5A-Tg mice as compared with wt mice. Because direct ex vivo quantification of NS5A-specific CD8+ T cells revealed a statistical significant difference between wt and NS5A-Tg mice (p < 0.001; Fig. 3E–I), the reason for the similar level of lytic activity detected in wt and NS5A-Tg mice can be explained by the 5-d in vitro restimulation with the peptides.

**In vivo protection against growth of NS5A-expressing tumor cells**

One way to evaluate the functionality of a vaccine-primed T cell response in vivo is to challenge immunized mice with syngeneic tumor cells expressing the vaccine Ag. Thus, determination of protection against tumor growth is a direct measure of in vivo T cell functionality (48, 49). We and others have shown that the protection against in vivo tumor growth is mainly dependent on cytotoxic T cells (CTLs) (44, 48). In this study, we used this tumor model to characterize in vivo functional immune responses to NS5A. To perform the proposed studies, we generated an EL-4 lymphoma cell line with a constitutively expressed NS5A protein by stable DNA transfection using the wtNS5A-pcDNA3.1 plasmid. The presence of HCV NS5A RNA in total RNA extracts from transfected EL-4 cells was analyzed by RT-PCR. Only extracts from the NS5A-EL-4 cell line were positive for NS5A by RT-PCR (Supplemental Fig. 1A). The NS5A-EL-4 and the parental EL-4 tumor cell models were evaluated in naive C57BL/6J mice to determine the specificity of protection against tumor growth. There was no statistical significant difference between the animals were sacrificed and immune responses were analyzed once with 50 μg full-length or truncated NS5A-DNA–based vaccine constructs (Table I). Only groups of C57BL/6J mice immunized with full-length wt NS5A plasmids or truncated constructs that contained the NS5A sequence between aa 216 and 449 were protected against NS5A-EL-4 tumor challenge. Moreover, only these groups had detectable NS5A2251–2259- and NS5A2252–2259-specific IFN-γ production (Fig. 5A, 5B). As expected, only plasmids containing the MHC class I NS5A epitopes (aa 281–289) induced IFN-γ production (Fig. 5B). In addition, mice immunized with NS5A region aa 302–449 were also protected against tumor growth (p < 0.05; Fig. 5A), even though this region does not contain the identified epitopes. This indicates the presence of additional CTL epitopes within NS5A in the H-2b haplotype.

**Expansion and polyfunctionality of the activated NS5A-specific T cells**

Because high quality polyfunctional CD8+ T cells are a hallmark of highly efficient human vaccines, we analyzed the polyfunctionality of T cell responses in wt and NS5A-Tg mice immunized once with 50 μg coNS5A-pVAX1 i.m. and EP. Two weeks later, the animals were sacrificed and immune responses were analyzed by ELISPOT, pentamer- and intracellular cytokine staining. The results revealed IFN-γ production in both wt and NS5A-Tg mice, although a statistically significantly higher number of SFCs was evident in the wt group (p < 0.001; Fig. 3A, 3B). The control groups were negative for IFN-γ production (Fig. 3C, 3D). Direct ex vivo pentamer staining was used to quantify the number of NS5A-specific CD8+ T cells after immunization. Quantifiable levels of NS5A-specific CD8+ T cells were detected in vaccinated wt and NS5A-Tg mice but not in nonimmunized groups. It was noticed that pentamer staining in NS5A-Tg mice was generally weaker, presumably because of the low frequency

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difference (e.g., wt mice compared with NS5A-Tg mice) has been indicated as follows: ***p < 0.001, **p < 0.01 using area under the curve (AUC) and ANOVA.
FIGURE 3. NS5A–DNA-based immunization primes NS5A-specific polyfunctional T cell responses in both wt and NS5A-Tg mice. Wt and NS5A-Tg C57BL/6J mice were immunized i.m. once with 50 μg CON5A-pVAX1, followed by in vivo electroporation. Two weeks postimmunization, mice were sacrificed, and quantification of NS5A-specific IFN-γ–producing T cells per 10^6 splenocytes was determined by ELISPOT assay (A–D). Data have been given as SFCs/10^6 splenocytes and SD. Cutoff has been set as 50 SFCs/10^6 splenocytes. Also shown is the expansion of NS5A-specific CTLs in wt and NS5A-Tg mice as determined by direct ex vivo pentamer staining, with data given as representative dot plots (E–H) and as the percentage pentamer-positive CD8+ T cells with SD (I). In (J) and (K), bars illustrate the percentage of CD8+CD107a+TNFα/IL-2/IFNγ–producing cells after 12 h ex vivo Ag stimulation.

Number of mice used in experimental setting: immunized wt mice (n = 10), immunized NS5A-Tg mice (n = 9), nonimmunized wt mice (n = 10), and nonimmunized NS5A-Tg mice (n = 8). The presence of a statistical difference (e.g., wt mice compared with NS5A-Tg mice) has been indicated as follows: ***p < 0.001, **p < 0.01, *p < 0.05 using area under the curve (AUC) and ANOVA (A, B) and a Mann–Whitney U test (e.g., all groups compared, I).
FIGURE 4. NS5A-DNA–based immunization primes NS5A–specific CTLs that protects against in vivo tumor growth. Mice were immunized i.m. once with 50 µg coNS5A-pVAX1 (A) or wtNS5A-pcDNA3.1 (B), followed by in vivo electroporation. Two weeks postimmunization, mice were challenged with NS5A-expressing EL-4 cells or with the parental EL-4 cells (1 × 10^6 cells s.c. in the right flank/mouse). Tumors were measured every second to third day with start at day 6 postinoculation. Values are presented as mean tumor volumes ± SE in groups of nine mice. Quantification of NS5A-specific IFN-γ–producing T cells per 10^6 splenocytes determined by an ELISPOT assay. Mice were immunized i.m. once with 50 µg coNS5A-pVAX1 (C, E), wtNS5A-pcDNA3.1 (D, F), or left nonimmunized (G). Two weeks postimmunization, mice were challenge using NS5A-EL-4 (C, D) or EL-4 (E, F) cells, and NS5A-specific IFN-γ–producing T cells were detected at the end of the experiment (n = 9). Data have been given as SFCs/10^6 splenocytes and SD. Cutoff has been set as 50 SFCs/10^6 splenocytes. Mice were immunized i.m. once with 50 µg coNS5A-pVAX1, followed by in vivo electroporation or left nonimmunized in wt mice (H, n = 10 mice/group) or NS5A-Tg mice (I, n = 8 mice per group). Two weeks postimmunization, mice were challenged with NS5A-expressing EL-4 cells (1.5 × 10^6 cells s.c. in the right flank/mouse). Tumors were measured at days 5, 7, and 9 postinoculation. Values are (Figure legend continues)
found in this group. Our results also indicate that the frequency of NS5A-specific CD8+ T cells was statistically significantly higher in wt mice (range, 0.7–3.6%) compared with NS5A-Tg mice (range, 0.2–0.7%) (p < 0.001; Fig. 3E–I). The polyfunctional activity of NS5A-specific T cells was determined by flow cytometry (Fig. 3J, 3K). Immunization of wt mice primed NS5A-specific T cells producing mainly combinations of IFN-γ/TNF-α/CD107a specific for the NS5A CTL epitopes. The expression pattern was similar in NS5A-Tg mice, albeit at lower levels. Interestingly, we could detect multifunctional T cells producing IFN-γ/IL-2/TNF-α/CD107a in both wt and NS5A-Tg mice. Thus, the NS5A-DNA immunization was highly efficient in activating NS5A-specific T cells to produce cytokines that enabled expansion and broadened the polyfunctional activity of the T cell response.

Discussion

The NS5A protein of HCV is an essential component of the replication complex (25, 26). The protein is subdivided into three distinct parts, domains I, II, and III, where domains I and II have been shown to be essential for RNA replication, whereas domain III is important for sequestration of infective virions (27). In addition, several studies have shown that NS5A may be a target for antiviral therapy. Results from clinical evaluation of NS5A-specific DAAs revealed that the candidate drugs had potent antiviral activity (21–23). The encouraging results suggest that NS5A-specific DAAs may be part of future antiviral therapy for HCV. Interestingly, combination treatment of HCV genotype 1 infection using NS3- and NS5A-specific DAAs achieved SVR without IFN-α and ribavirin, although the addition of IFN-α and ribavirin produced superior responses (50). With this in mind, we decided to pursue a detailed characterization of NS5A as a genetic vaccine candidate for chronic HCV infection. Because of the limited knowledge of the immunogenic properties of NS5A, we sought to study its intrinsic properties. The limited number of studies of the intrinsic properties of NS5A as a genetic immunogen has demonstrated poor immunogenicity (17–19). Moreover, only a limited number of HLA-A2 CD8+ T cell–restricted epitopes have been identified (16, 51, 52). We therefore cloned full-length and truncated NS5A genotype 1b genes into expression vectors and evaluated the immunogenicity in wt and NS5A-Tg mice. In this study, we demonstrated that the carboxyterminal domains II and III of NS5A are important for priming NS5A-specific humoral immune responses. It is known that this region of NS5A contains several human B cell epitopes (53, 54). Our results show that a NS5A protein was more immunogenic in priming NS5A-specific IgG Abs compared with NS5A as a DNA plasmid. Although, the ability of NS5A DNA to elicit NS5A-specific IgG Ab titers of >104 postimmunization highlights that NS5A is intrinsically immunogenic and may be suitable in vaccine compositions. To be able to characterize cellular immune responses to NS5A in H-2b mice, we first had to identify MHC class I epitopes within NS5A. Several MHC class I epitopes are described in other mouse haplotypes (17–19), but to our knowledge, none is described for H-2b. We therefore used overlapping peptides covering the complete NS5A gene combined with epitope predictions. By fine-mapping of epitopes, we were able to identify two NS5A MHC class I epitopes sharing the same sequence but with different MHC restrictions (H-2Kb and H-2Db). Identified epitopes were tested for recognition by vaccine-primed CD8+ T cells and whether MHC–TCR recognition induced IFN-γ and IL-2 production in splenocytes from wt and NS5A-Tg mice. Before evaluating NS5A-specific immune responses in H-2b NS5A-Tg mice, we first confirmed liver-specific NS5A protein expression by Western blot analysis. The m.w. of the NS5A protein band was identical to that previously shown in NS5A-Tg FVB/N mice (20). We discovered that NS5A-specific immune responses could be activated in both wt and NS5A-Tg mice, although the Tg mice were tolerant as evidenced by significantly lower levels of IFN-γ and IL-2 produced after immunization. Importantly, the vaccine-primed T cells were able to kill peptide-pulsed target cells in a cytotoxicity assay. This proves that the activated T cells are functional. Similar results have been described in wt and NS3/4A-Tg mice immunized with a NS3/4A-based DNA vaccine (10). To further evaluate the in vivo functional characteristics of NS5A vaccine–primed immune responses, we established a homologous in vivo tumor challenge model in which NS5A is stably expressed in the EL-4 T cell lymphoma cell line. Parental EL-4 and NS5A-transfected EL-4 tumor cells had comparable growth properties in wt mice. To use this tumor challenge model, we first determined the specificity of tumor protection by challenging immunized mice with the transfected tumor cells or the parental cell line and comparing in vivo protection. We showed that only NS5A-immunized groups of mice were protected against tumors expressing the NS5A protein. Similar specificity of protection has been shown in other tumor challenge models (44). In addition, the protection is mainly confirmed by CD8+ T cells and to a lesser extent CD4+ T cells (44). Interestingly, protection against tumor growth in immunized NS5A-Tg mice was not complete although significant compared with nonimmunized NS5A-Tg mice. This demonstrates that even in a tolerogenic environment, as in NS5A-Tg mice, dysfunctional T cells can be activated by NS5A–DNA immunization and mediate protection. Hence, a single NS5A–DNA immunization in NS5A-Tg mice can prime T cells that inhibit NS5A-expressing tumor cells. Moreover, analysis of immune responses in immunized and NS5A-EL-4 tumor challenged mice revealed an improved immunogenicity as compared with immunization and EL-4 tumor challenge. Thus, the improved immunogenicity may be explained by a boost from the NS5A protein expressed in the tumor cells. We have previously seen similar results in a hepatitis B virus tumor model (55). We were also interested in the importance of the different regions and domains of NS5A. Several studies have shown that the three domains of NS5A have important functions in the viral life cycle (27–32). Simultaneously, we were interested whether the different regions and domains also had different immunogenic properties. This was investigated by testing eight truncated NS5A constructs for priming protective immunity against tumor challenge using EL-4 cells stably expressing the full-length NS5A-protein. This revealed that region aa 216–449 was important for protection against NS5A-EL-4 tumor growth. Analysis of immune responses in the same mice showed that all constructs containing the NS5A CTL epitopes elicited IFN-γ production. In addition, one construct that did not contain the NS5A CTL epitopes also elicited protection against tumor growth, indicating that protection is not solely dependent on immune responses to the identified CTL epitopes. Hence, our results indicate that, at least in mice, the minimum region of NS5A needed to mediate protection against tumor growth and activate NS5A-specific T cell responses is the...
region connecting domains I and II, and the amino terminal part of domain II.

One important feature for a therapeutic vaccine candidate is to prime high frequencies of lytic T cells that can also produce multiple cytokines. We therefore analyzed the polyfunctionality of T cells primed in wt and tolerant NS5A-Tg mice. We showed that NS5A vaccination in wt and NS5A-Tg mice primed NS5A-specific T cell responses with frequencies of $\sim 1.5\%$ NS5A-specific CD8$^+$

**FIGURE 5.** Immunization with truncated NS5A-DNA immunogens mediates protection against in vivo tumor growth. Mice were immunized i.m. once with 50 $\mu$g truncated wtNS5A expression plasmids (see Table I), followed by in vivo electroporation. Two weeks postimmunization, mice were challenged with NS5A-expressing EL-4 cells ($1 \times 10^6$ cells s.c. in the right flank/mouse). Tumors were measured every second to third day with start at day 5 postinoculation (A). Values are presented as mean tumor volumes ± SE in groups of seven mice. Quantification of NS5A-specific IFN-γ-producing T cells per $10^6$ splenocytes determined by an ELISPOT assay at the end of the experiment (B). Data have been given as SFCs/$10^6$ splenocytes and SD. Cutoff has been set as 50 SFCs/$10^6$ splenocytes. Truncated constructs 1–163, 1–215, and 302–449 do not contain the sequences for the identified CTL epitopes and was therefore negative for IFN-γ production in response to peptide stimulation. The presence of a statistical difference (e.g., NS5A-based plasmid compared with pcDNA3.1 empty vector) has been indicated as follows: ***$p < 0.001$, **$p < 0.01$ using area under the curve (AUC) and ANOVA.
T cells in wt mice compared with 0.5% in NS5A-Tg mice. This further highlights the tolerant status of these Tg animals but also shows that vaccination can activate an immune response. Importantly, when determining the pattern of cytokines produced after immunization, we found a similar expression profile in wt and NS5A-Tg mice. This may be compared with the cytokines produced by peptide-stimulated TCRs with affinities for cognate ligands below the threshold required for peptide–MHC pentamer engagement. Thus, CD8+ T cells may exhibit effector functions in the absence of detectable pentamer binding. In addition to intrinsic affinity, variations of TCR density, differences in membrane lipid organization, the state of T cell activation, and differentiation status may also influence pentamer binding (56–60). Therefore, peptide stimulation used in functional assays enhances the detection of pentamer-negative functional T cells. We demonstrated that NS5A-based DNA vaccines were able to prime specific T cell responses of suitable phenotype. Therefore, NS5A may be considered as a therapeutic vaccine candidate for chronic HCV infection. Moreover, even though NS5A may interfere with cellular signaling pathways including molecules and cells involved in innate and adaptive immunity (20, 34, 35), long-term intrahepatic NS5A protein expression does not induce any liver disease (20, 36).

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Disclosures
L.F. and G.A. are paid consultants to ChronTech Pharma AB. L.F. owns patent/patents pending for HCV Tg mouse models and HCV therapeutics. K.E.B. is an employee of Inovio Pharmaceuticals. The other authors have no financial conflicts of interest.

References
Supplementary figure 1. *In vivo* growth of NS5A stable expressing EL-4 cells.

Detection of NS5A mRNA in stable transfected EL-4 cells by reverse transcriptase (RT)-PCR (A). Lane 1: NS5A transfected EL-4 cells, lane 2: non-transfected EL-4 cells, lane 3: H2O RT-PCR control, lane 4: H2O PCR control, lane 5: negative PCR control, and lane 6: positive PCR control. Samples in lane 1-3 were run with RT polymerase. Lane 7-12: doublets of the same samples as 1-3 but without RT polymerase in the RT reaction. M: size marker GeneRuler™ 100bp (Fermentas, Leon-Rot, Germany). Expected DNA band sizes: NS5A 550 nt and mouse tubulin 350 nt. B) Comparison of *in vivo* growth properties of NS5A-EL-4 and EL-4 cell lines in C57BL/6J mice inoculated with 1x10^6 tumor cells (n = 9). Values are presented as tumor volume for single mice measured every second to third day with start at day six post tumor injection. Statistical analysis was performed using AUC and ANOVA and compared tumor growth in groups of wildtype and NS5A-Tg mice.
Supplementary fig. 1