CHAPTER III

MATERIALS AND METHODS

Study population

In the comparative study of different genotypic methods, thirty-five plasma samples were obtained from ten chronic HCV patients from the Erasmus MC, Rotterdam, The Netherlands, seven chronic HCV patients from Phra Mongkutklao Hospital, Bangkok, Thailand and 18 HCV-RNA positive blood donors from the National Blood Centre, Thai Red Cross, Bangkok, Thailand.

For study of distribution of HCV genotypes in Thailand and HCV immune response, whole blood samples screened positive for anti-HCV were collected anonymously from new blood donors at the National Blood Centre, Thai Red Cross, Bangkok, Thailand, conducted from June 2001 to January 2002. Ten healthy donors without a clinical history of hepatitis, symptoms or signs of liver disease and negative for anti-HCV were used as negative controls.

The project was approved by the Ethics Committee, Ministry of Public Health and Faculty of Medicine, Chulalongkorn University.

Materials

1. Cell lines : NKNK-3 (kindly provided by Dr. Bart L. Haagmans)
2. E.coli, NVT4 (Invitrogen, USA)
3. pCR2.1 vector (TA cloning kit, Invitrogen, USA)
4. VR 1012 mammalian expression plasmid (Vical Incorporated, USA)
5. pNS (EBV-based) plasmid (kindly provided by Dr. Bart L. Haagmans)
6. HCV-SOD antigens (derived from HCV 1a genotype):
   - SOD-SDS (control human superoxide dismutase)
   - SOD-c22-3 (core : amino acid 2-120)
   - SOD-c200 (NS3/4 : amino acid 1192-1931)
   - SOD-NS5 (NS5 : amino acid 2054-2995)

(kindly provided by Chiron Corporation : Emeryville, CA)
7. Vaccinia virus wild type (kindly provided by Chiron Corporation: Emeryville, CA)

8. Recombinant vaccinia viruses expressing HCV genes derived from HCV 1a genotype (kindly provided by Chiron Corporation: Emeryville, CA)
   - vv-poly (amino acid 1-966)
   - vv-NNRd (amino acid 364-1619)
   - vv-NS4 (amino acid 1590-2053)
   - vv-NS5A (amino acid 2006-2397)
   - vv-NS5B (amino acid 2396-3011)

   (the diagram of all vaccinia virus vectors have been shown in Figure 6)

9. EBV supernatant

10. PCR purification kit (QiAquick®, Qiagen, Germany)

11. StrataPrep EF Plasmid Midiprep kit (Stratagene, USA)

12. PRISM Ready Reaction Dye Deoxy Terminator cycle sequencing kit (Applied Biosystems, USA)

13. ELISPOT reagent kit for detection of IFN-γ production (Mabtech AB, Sweden)

14. Cytofix/Cytoperm Plus™ (with GolgiStop™) (Pharmingen, San Diego, CA)

15. Perfectprep Gel Cleanup Kit (Eppendorf, Westbury, NY)

16. MicroAmp PCR tube (Perkin Elemer, USA)

17. Microcentrifuge tube: 0.5 and 1.5 ml. (AxyGen® Scientific, USA)

18. Polypropylene conical tube: 50 and 15 ml. (AxyGen® Scientific, USA)

19. Pipette tip: 10 μl, 200 μl and 1000 μl (AxyGen® Scientific, USA)

20. Cryotube (Nunc, USA)

21. Microscope slide and cover slit (Sail brand, China)

22. Glassware: Beaker, Flask, Cylinder and reagent bottles (Pyrex, USA)

23. Tissue Culture Flask, Culture plate, Sterile serological pipette 10, 5 and 1 ml (Costar, USA)

24. Cell strainer 100 μM nylon and polystyrene plate (Becton Dickinson, USA)

25. Counting chambers

26. Nitrocellulose-bottom Silent Screen Plate 96-well (Nalge Nunc International)
27. INNO-LiPA HCV II kit (Innogenetics N.V., Ghent, Belgium)
28. INNO-LiPA HCV II Amplification kit (Innogenetics N.V., Ghent, Belgium)

Equipments

1. Centrifuge (Beckman GS-6R, USA)
2. Refrigerated microcentrifuge (Universal 16R Hettich, USA)
3. – 70 °C freezer (Forma Scientific, USA)
4. – 20 °C freezer (Philco, USA)
5. Light microscopy (Nikon Y52, Japan)
6. DNA Thermal Cycler 9600 (Perkin Elmer, USA) and Mastercycler personal (Eppendorf) (Axygen, USA)
7. Gel Doc 1000 UV transilluminator (Biorad, USA)
8. Mitsubishi video copy processor (Mitsubishi, Japan)
9. Ultrahigh speed centrifugation (55p-72 HIMAC Centrifuge Hitachi, Japan)
10. Stereo microscope (Nikon, Japan)
11. Spectrophotometry (Shimadzu UV-160A, Japan)
12. Perkin-Elmer 310 Sequencer (PE Biosystems, USA)
13. CO₂ humidified incubator (TC2523 Shelloab, USA)
14. FACScan analyzer (Becton Dickinson, USA)
15. Autoclave (Hydroclave MC10 Harvey, USA)
16. Hot air oven (Memmert, West Germany)
17. Multi-block heater (Lab-line, USA)
18. Balance (PB1502 Mettler Toledo, Switzerland)
19. β scintillation counter
20. Irradiated machine (Gamma cell 40 atomic energy, Canada)
21. Gene pulser apparatus (Biorad, CA)
22. Gene Pulser® Cuvette (Biorad, CA)
23. Microwave oven (Sanyo, Japan)
24. Multiwell harvester
Reagents

1. Phenol (Sigma, USA)
2. Chloroform (Merck, USA)
3. Isoamyl alcohol (Merck, USA)
4. Sodium acetate (Sigma, USA)
5. Absolute ethanol (Merck, USA)
6. Isopropanol (Merck, USA)
7. Reagents for PCR analysis
   10x PCR buffer (Finnzymes, Finland)
   Deoxynucleotide triphosphate (dNTPs) (Promega, USA)
   Taq DNA polymerase (DyNAzyme™ II DNA Polymerase, Finnzymes, Finland)
8. Reagents for cDNA synthesis
   5x M-MLV RT buffer (Promega, USA)
   M-MLV Reverse Transcriptase (Promega, USA)
   RNasin® Ribonuclease Inhibitor (Promega, USA)
9. Restriction enzymes: Ava I, Sma I and Mbo I (New England Biolabs, USA)
10. Agarose gel (FMC Bioproducts, USA)
11. NuSieve agarose (FMC Bioproducts, USA)
12. Ethidium bromide (Sigma, USA)
13. Template suppression reagent (TSR) (Applied Biosystem, USA)
14. Guanidinium thiocyanate (GTC) (USB, USA)
15. 2-Mercaptoethanol (2-ME) (Sigma, USA)
16. Glycogen (Sigma, USA)
17. Diethylpyrocarbonate (DEPC)
18. X-gal (Biobasic Inc., Germany)
19. IPTG and ampicillin (Biobasic Inc., Germany)
20. Streptavidine (Amersham, Pharmacia Biotech, USA)
21. 3,3′,5,5′-tetramethyl-benzidene (TMB) substrate (Amersham, Pharmacia Biotech, USA)
22. Amino-9-ethycarbazole (AEC) (Sigma, USA)
23. Anti-mouse Ig – biotin/avidin HRP (Amersham, USA)
24. Monoclonal antibody to NS3 protein (5F-1, Organon)
25. 5-bromo-4-chloro-3-indolyphosphate/nitroblue tetrazolium substrate
   (BCIP/NBT) (Biorad, USA)
26. RPMI-1640 medium (Gibco BRL, USA)
27. Fetal bovine serum (Gibco BRL, USA)
28. L-Glutamine (Gibco BRL, USA)
29. Normal goat serum (Gibco BRL, USA)
30. Penicillin/ Streptomycin (Gibco BRL, USA)
31. Geneticin G418 (Sigma, USA)
32. Collagenase/Dipase (Sigma, USA)
33. Dnase I (Gibco BRL, USA)
34. Trypsin (Gibco BRL, USA)
35. Glycine (Merck, USA)
36. Trypan blue (Sigma, USA)
37. H₂O₂ (Sigma, USA)
38. Hepes (Merck, USA)
39. NaCl (Sigma, USA)
40. KCl (Sigma, USA)
41. Na₂HPO₄ (Merck, USA)
42. CaCl₂ (Merck, USA)
43. PBS (Sigma, UK)
44. Na₂CO₃ (Sigma, UK)
45. NaHCO₃ (Sigma, UK)
46. NaN₃ (Sigma, UK)
47. KH₂PO₄ (Sigma, UK)
48. MgCl₂ (Sigma, UK)
49. Sodium-N-Lauroyl-sarcosinate (C₁₅H₂₆NNaO₃) (Sigma, UK)
50. Phytohemagglutinin (PHA) (Sigma, UK)
51. Interleukin-2 (IL-2) (Genzyme, USA)
52. Lymphoprep™ (NYCOMED PHAMA AS, Oslo, Norway)
53. Tween 20 (Sigma, UK)
54. Pooled human serum
55. Dimethyl sulfoxide (DMSO) (Sigma, UK)
56. "H-Thymidine (Amersham Biosciences, Sweden)
57. LB medium (Gibco BRL, USA)
58. Cyclosporin A (Sigma, UK)
59. Anti-CD3-Cy5, anti-CD4-FITC, anti-CD8-FITC, anti-IFN-γ-PE, isotype control-PE, anti-mouse Ig (DAKO A/S, Denmark)
60. Lipo-fectamine™ 2000 Reagent (Gibco BRL, USA)

Software and program for phylogenetic analysis
1. Clustal X program, version 1.4
2. PHYLIP package, version 3.57c (J. Felsenstein, Department of Genetics, University of Washington): SEQBOOT program, DNADIST, NEIGHBOR and CONSENSE software
3. TREEVIEW program, version 1.5
4. Cell Quest software (Beckton Dickinson, San Jose, CA)

Methods
1. Anti-HCV serological test
   Anti-HCV serology was performed using a commercially available third generation ELISA test kit (Abbott Laboratory, North Chicago, Ill) according to the manufacturer's recommendations. This test was performed by The National Blood Center, Thai Red Cross.

2. Plasma collection
   Plasma was separated from whole blood by centrifugation at 2,000 rpm for 10 min and kept at -70°C for further analysis (RT-PCR for HCV-RNA and HCV genotyping).
3. PBMC isolation

Peripheral blood mononuclear cells or PBMCs were separated from whole blood, using Lymphoprep™ by centrifugation at 2,800 rpm for 30 minutes. Cells were then washed with PBS for 3 times, cryopreserved in freezing medium (Appendix A), kept at –70 °C for one night before transferring to liquid N₂ and storage until used for HCV immune response study.

4. RNA preparation, c-DNA synthesis and nested PCR amplification

The viral RNA was extracted directly from plasma by guanidinium method \(^{(146)}\). One hundred μl of each sample was mixed with 500 μl GTC-2ME (Appendix A). After inverted mix and vortexing, RNA was extracted by the mixture of 50 μl 2M sodium acetate, 500 μl phenol, 100 μl chloroform:isoamyl alcohol (49:1), vortexed for 10 seconds and cooled on ice for 15 minutes. The sample was centrifuged at 14,000 rpm for 20 minutes at 4°C. The aqueous phase which contain RNA was transferred to a new tube, mixed with 4 μl glycerogen and 500 μl isopropanol and then placed in –20 °C freezer for at least 3 hours to precipitate RNA. Sedimentation of RNA was performed by centrifugation at 14000 rpm for 20 minutes at 4°C. RNA pellet was washed twice with 1 ml of precooled 70% ethanol. The supernatant was discarded and RNA pellet was allowed to air dry. The RNA pellet was resuspended in 10 μl DEPC (diethylpyrocarbonate) treated sterile water and directly used as a template for RT-PCR. The RNA was heated to 65 °C for 5 minutes, cooled on ice and incubated in a reaction mixture containing 1x RT buffer, 0.3 μM primer \(^{(410)}\), 0.5 mM dNTPs, 20 U Rnasin \(^{®}\) ribonuclease inhibitor and 100 U M-MLV reverse transcriptase at 37 °C for 1 hour to generate cDNA.

cDNA (5 μl) was amplified by nested PCR as followed: The nested PCR of core gene was amplified for HCV DNA screening by 2 sets of primers. \(^{(10,13)}\) Primers 410 and 954 were used for the first amplification round and primers 951 and 953 in the second, respectively. The details and sequences of primers are described in Table 4. The amplification cycle required 35 cycles comprising an initial step at 95 °C for 3 minutes, denaturation at 94 °C for 1 minutes, annealing at 48 °C for 2 minutes, extension at 72 °C for 2 minutes, concluded by a final extension step at 72 °C for 7 minutes in an automated
thermocycler (Eppendorf). The 405 bp PCR products between positions –21 and 383 were analyzed by electrophoresis in a 1.5% agarose gel stained with ethidium bromide and visualized on a UV transilluminator.

<table>
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<tr>
<th>Primer</th>
<th>Length</th>
<th>sequences (5’ → 3’)</th>
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<th>usage</th>
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<td>ATGTACCCCATCAGGTCCGC</td>
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<td>core</td>
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<td>954</td>
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<td>ACTCCCTGATACGCTCGCTGAG</td>
<td>-54(-31)</td>
<td>outer forward</td>
<td>core</td>
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<td>383-364</td>
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<td>core</td>
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<td>core</td>
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<td>AGGTTGGGTTCAGGGCAAT</td>
<td>4858-4834</td>
<td>reverse</td>
<td>NS3</td>
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5. HCV genotyping methods

Thirty-five samples described in study population were used. Four methods for HCV genotyping according to core region of viruses, RFLP using two different set of restriction enzymes, direct sequencing and INNO-LiPA assay were performed.

5.1 Restriction Fragment Length Polymorphism (RFLP)

The 405 bp amplified product of core gene was genotyped by RFLP using two different sets of restriction enzymes and conditions. The first one, restriction enzymes Acc I, Mbo I and BstN I were used as described by Buoro et al. The second one, a method described by Mellor et al., using the restriction enzymes Ava I and Sma I was used. Because different primers were used, shorter PCR products and also slightly different.

The experiments were done, with lightly modification from previously reports, as followed, a volume of 15 µl of PCR product was mixed with 2 µl of 10xbuffer, 10 units of each enzyme and adjust volum to 20 µl with water. After incubation at optimal temperature for each enzyme for 4 hours, the samples were analyzed by electrophoresis
using 3% NuSieve agarose gel (3:1), stained with ethidium bromide and visualized on a UV transilluminator.

The expected RFLP electropherotype patterns should be obtained from RFLP using both conditions were shown in Table 5 and 6. The interpretation of HCV genotypes of samples were derived by comparing with the expected restriction endonuclease patterns shown in Table 5 and 6.

Table 5  Electropherotypes expected from Acc I, Mbo I and BstN I digestion on 405 bp fragment.

<table>
<thead>
<tr>
<th>Type on 1a</th>
<th>1b</th>
<th>1c</th>
<th>2a</th>
<th>2b</th>
<th>2c</th>
<th>3a</th>
<th>3b</th>
<th>4a</th>
<th>5a</th>
<th>6a</th>
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<td>Acc I</td>
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<td>Mbo I</td>
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Table 6  Electropherotype patterns from Ava I and Sma I digestion of the 405 bp core fragment

<table>
<thead>
<tr>
<th>Pattern</th>
<th>Fragment length (bp)</th>
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<tbody>
<tr>
<td>A1*</td>
<td>148 101 94 38 24</td>
</tr>
<tr>
<td>A2</td>
<td>148 101 94 62</td>
</tr>
<tr>
<td>A3</td>
<td>148 139 94 24</td>
</tr>
<tr>
<td>A4</td>
<td>172 139 94</td>
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<td>A5</td>
<td>139 94 52 24</td>
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<td>A6</td>
<td>233 148 24</td>
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<td>A7</td>
<td>148 94 87 52</td>
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<td>172 94 87 52</td>
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<td>172 101 94 38</td>
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<td>163 148 94</td>
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<td>260 94 52</td>
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<td>A12</td>
<td>311 94</td>
</tr>
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<td>A13</td>
<td>210 101 94</td>
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<table>
<thead>
<tr>
<th>Pattern</th>
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<td>S1*</td>
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<tr>
<td>S4</td>
<td>148 111 94 52</td>
</tr>
<tr>
<td>S5</td>
<td>311 94</td>
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</table>

* A=Ava I  S=Sma I

Interpretation:

<table>
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<tr>
<th>Genotype</th>
<th>common patterns</th>
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<tr>
<td>1a</td>
<td>A1S1, A2S1</td>
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<tr>
<td>1b</td>
<td>A3S1, A4S5</td>
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<tr>
<td>6a</td>
<td>A12S5</td>
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</table>
5.2 Sequencing and phylogenetic analysis

The 405 bp PCR product of core region were purified from agarose gel for sequencing using the Perfectprep Gel Cleanup Kit, according to the manufacturer's specifications, and subjected to 1.5% agarose gel electrophoresis in order to ascertain their purity.

Concentration of the amplified DNA was determined by measuring the absorption of every sample at 260 nm in a UV spectrophotometer. The concentration was calculated according to the formula 1 OD 260 = 50 μg double-stranded DNA. Between 10 and 30 ng/μl (3-6 μl) of each DNA sample were subjected to cycle sequencing using 8 μl of dye terminator from a DNA sequencing kit and 3.2 pmole of specific primer (in a final reaction volume of 20 μl) in a thermocycler. This round of amplification was performed according to the manufacturer's specifications, using upstream primer 953 and reconfirmed by using downstream primer 951 to amplify the particular DNA strand of interest for further sequencing. The extension products were subsequently purified from excess unincorporated dye terminators by ethanol precipitation, according to the manufacturer's specifications and subjected to sequence analysis by ABI Prism 310 Genetic Analyser. Regarding the rest of the subsequent steps, we referred to the ABI Prism 310 Genetic Analyser user's manual.

Nucleotide sequences were multiplied and aligned with Clustal X program, version 1.4. Bootstrap analysis was performed for values representing 1,000 replicates by SEQBOOT program. Distances between pairs of sequences were estimated by DNADIST program of PHYLIP package (version 3.5c). The distances were clustered into phylogenetic groupings by NEIGHBOR and CONSENSE softwares from PHYLIP package. Equivalent phylogenetic relationships were also found in the maximum likelihood analysis. TREEVIEW program, version 1.5, was run for phylogenetic tree construction.

New sequences obtained in the study have been submitted to GenBank and have been assigned accession numbers as shown in Appendix C. The genomic sequences of 11 different HCV strains were obtained from GenBank to be used as reference standard sequences for phylogenetic tree. The accession numbers were namely: AF 387806(1a), AF 333324(1b), D 14853(1c), D 00944(2a), D 10988(2b), AY
070175(2c), D 17763(3a), D 49374(3b), Y 11604(4a), Y 13184(5a), Y 12083(6a). HCV sequences on the same node were interpreted to belong to the identical genotypes.

5.3 INNO-LiPA assay

In this procedure, labeled PCR products obtained from the 5' NCR (from INNO-LiPA™ HCV II Amplification kit) were hybridized to immobilized oligonucleotide probes which were specific for the six major types and can identify most subtypes. The principle of this test and also the structure of the strip are shown in Figure 5.

![Figure 5 Principle of INNO-LiPA method (left) and the strip for result interpretation (right)](image)

6. HCV-specific lymphocyte proliferation assays

Proliferation assays were performed as follows. PBMCs (2x10^5 cells) in R10F (Appendix A) were cultured in 96 wells round bottom microplates in the presence or absence of HCV recombinant proteins; SOD-SDS, SOD-c22-3 (core), SOD-c-200 (NS3/4) and SOD-NS5 at 3 μg/ml final concentrations. After 6 days, 1 μCi of ³H-Thymidine was added in each well and radioactivity of incorporated DNA was measured after an additional 16 hours by liquid scintillation counting. Proliferation was considered positive when stimulation index (cpm obtained in the presence of antigen divided by cpm obtained in the absence of antigen) was ≥ 3 and Δ cpm (the difference between
cpm obtained in presence of antigen and cpm obtained in the absence of antigen) was > 2000.

7. Detection of IFN-γ production

Two methods were used for detection of IFN-γ in this study.

7.1 ELISPOT method

Nitrocellulose-bottom Silent Screen Plate 96-well (Nalge Nunc International) were coated with 100 µl of the IFN-γ monoclonal antibody at the concentration of 1 µg/ml in 0.1 M carbonate bicarbonate buffer pH 9.6 and were incubated overnight at 4°C. Unbound antibodies were removed via 3 successive washings with PBS. The coated wells were blocked with R10H culture medium (Appendix A) for 2 hours in 37°C incubator. After that the wells were duplicated with 100 µl of R10F containing 1×10⁵ T cells or 2×10⁵ PBMCs together with indicated stimuli, or a positive control, PHA(1µg/ml). The mixtures were incubated for 24 hours at 37°C in humidified CO₂ incubator.

After incubation, the cells were removed by washing the plate 6 times with PBS containing 0.05% Tween20. 100 µl of the biotin-conjugated anti-IFN-γ was added to each well at the concentration of 1 µg/ml and incubated for 3 hours at room temperature. The plates were rinsed 3 times by immersion in PBS containing 0.05% Tween20 and were exposed to 100 µl of streptavidin-alkaline phosphatase (Mabtech AB) for 1 hour. Unbound conjugate was removed by washing thoroughly with PBS, and finally, 100 µl of 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium substrate solution was added, and the sample was incubated for 40 minutes. The color reaction was stopped by extensive washings 3 times with distilled water, and after drying, the number of spots was scored by use of a dissection microscope.

For detection of IFN-γ production by PBMCs induced by HCV proteins, 2×10⁵ PBMCs were incubated with indicated HCV-SOD proteins (or negative, positive control) for 24 hours in round bottom 96 well culture plate and transferred into the ELISPOT plate that had already coated with anti-IFN-γ for overnight at 4 °C and the assay was further performed as described above.
For detection of IFN-γ production induced by BLCLs that present HCV proteins, 1x10^5 T cells or 2x10^5 PBMCs were incubated with 2x10^4 inactivated BLCLs presenting HCV proteins (or medium with PHA as positive control) in a round bottom 96 well plate in 37°C incubator for 4 hours before transferring to anti-IFN-γ-coated plate. Detection of anti-IFN-γ production was continued as described above.

7.2 Intracellular IFN-γ detection using FACS analysis

Liver-derived T cell lines (2x10^6 cells) were resuspended in 2 ml of R10F and plated in 6 wells plate and stimulated for 2 hours with autologous BLCLs infected with recombinant vaccinia-HCV, autologous BLCLs stably transfected with pNS-HCV and control autologous BLCLs. The Golgi-Stop was added at the ratio of 4 µl Golgi Stop/6 ml medium and further incubated for 4 hours. The cells from each well were washed with staining buffer (Appendix A) for 2 times, resuspended in 150 µl staining buffer and then divided into 3 tubes. Cells in each tube were then stained for T cell surface markers and intracellular IFN-γ production. Briefly, the cells in the first tube were incubated with anti-CD3-Cy5 and anti-CD4-FITC, the second were incubated with anti-CD3-Cy5 and anti-CD8-FITC and the third one an isotype control for intracellular IFN-γ staining was used. After that cells were washed 2 times by 1 ml staining buffer and resuspended in Cytifix/Cytoperm solution for 20 minutes at 4°C. The permeabilized cells were washed 2 times with 1x Perm/Wash solution (Pharmlingen) and stained for intracellular IFN-γ production by using anti-IFN-γ-PE for 30 minutes in the dark and on ice. After this the cells were washed 2 times with 1x Perm/Wash solution and resuspended in 250 µl staining buffer before FACS analysis. FACS analysis was performed by FACSscan flow cytometers and analyzed with Cell Quest software.

8. Isolation of liver infiltrating Lymphocytes

Needle liver biopsies obtained from 7 chronically HCV-infected patients at outpatients clinic, Dijkzigt Hospital, Rotterdam, The Netherlands were washed extensively in phosphate-buffer saline (PBS) to remove contaminating blood. To disrupt the hepatic tissue and to release infiltrating mononuclear cells, liver specimens were minced by scalpel and then digested with 0.5 mg/ml collagenase/dipase enzymes and 40 KU/ml
Dnase I for 30 minutes in 37 °C incubator. The suspension was filtrated through a cell strainer (Becton Dickinson) and centrifuged at 1,600 rpm for 10 minutes. After discharging the supernatant, the pellet was resuspended and cultured in tissue culture medium (R10F) in the presence of irradiated allogeneic PBMCs, irradiated BLCLs, 1 μg/ml PHA and 50 IU/ml IL-2. Culture was maintained until cells stopped proliferating and were subsequently tested with specific antigens.

9. EBV-transformed B cells

BLCLs were established through Epstein-Barr virus (EBV) transformation and maintained in R10F medium. Briefly, 1×10⁶ PBMC cells were place into a 50 ml conical tube. The culture supernatant containing EBV was added, incubated for 2 hours in 37°C humidified CO₂ incubator. After adding R10F containing 1 μg/ml cyclosporin A, the cell suspension were transferred to a well of 6-well plate. When the colour of the medium turn to yellow, these cells were transferred to 25 cm² tissue culture flask containing 5 ml fresh R10F and further incubated for 1-2 weeks in humidified CO₂ incubator. The cell line was maintained by splitting 1:3 in R10F once a week until used.

10. Selection of HCV genome region for plasmid construction

NS3 region was chosen for plasmid construction because HCV specific response for this region was observed in one specific patient. Liver-infiltrating lymphocytes (LiLs) of this patient in particular (816) had strong HCV specific responses to the NN0d region when a biopsy was taken and tested. We decided to use LiLs of 816 and antigen in NN0d region as a model to test the efficiency of pNS vector in expression of HCV protein when compare with recombinant VV-HCV (rVV-HCV). When analysing the HCV region present in the different VV-HCV carefully, NN0d region (shown in Figure 6) was chosen to clone into the plasmid. The length of this region was approximately 1,980 bps and contained the NS3 protein to be used as expression marker and immunodominant epitopes.
11. Cloning of NNRd region

The 1,980 bp located in NNRd region (aa 966-1,619) was amplified from vaccinia-NNRd using forward primer (NNRdF) and reverse primer (NNRdR) (Table 4). The amplified PCR products were ligated into pCR2.1 vector (TA cloning® kit, Invitrogen) at the TA cloning sites using T4 DNA ligase according to the manufacturer's protocol. The ligated products were transformed into E.coli INVαF'. The clones were selected by X-gal/IPTG and ampicillin resistance. Recombinant plasmid was purified and inserted DNA was cut with the restriction enzyme EcoRI followed by a fill-in and dephosphorylation step. DNA was subcloned into a VR1012 Neo mammalian expression vector (VRNeo™, modified from VR1012 Vical by Noppompanth S.) at the EcoR V site and pNS EBV-based vector at the BamHI site and transformed into E.coli INVαF'. Kanamycin resistant clones were selected and plasmid DNA were extracted and examined by restriction enzyme digestion. Recombinant DNA was purified for the transfection experiment using the StrataPrep EF plasmid Midiprep Kit according to the manufacturer's protocol.
12. Transfection

NKNT-3 cells cultured in R10F were seeded on 6 well plate at the concentration 5x10^5 cells per well. Culture was kept at 37°C in humidified CO2 incubator for 24 hours. Twenty micrograms of purified vectors were transfected into these cells using Lipofectamine 2000 (LF2000) reagent according to the manufacturers' protocols.

7x10^6 BLCLs were resuspended in 200 µl K-PBS buffer (Appendix A)^{136,147} Purified pNS and pNS/N16 vectors were also resuspended in 100 µl K-PBS buffer and mixed individually to each tube of cells. The cell mixture was transferred to electroporation cuvette and pulsed at 390 V/500 µF in Gene Pulser apparatus. After the electroporation, cells were chilled on ice for 10 minutes and transferred to 25 cm^3 flasks that contain 7 ml fresh R10F medium. After 3 days following transfection, the cells were selected for plasmid expression by transferred to a new medium that contained 1 mg/ml G418 sulfate every 3 days for 3 times until tested with PBMC or NS3 expression.

13. Tests for NS3 protein expression

13.1 Immunostaining

Transfected NKNT-3 cells were cultured for 24-48 hrs and fixed with 5% paraformaldehyde in phosphate-buffered saline (PBS). After washing with PBS, cells were incubated with 1.0 M glycine for 10 minutes followed by 0.05% H2O2 in 70% ethanol. Non-specific binding was blocked with 1% normal goat serum. The mouse monoclonal antibody to NS3 (5F-1) was bound to transfected cells at 4°C for 12 hrs. A signal was detected by incubating with biotin conjugated goat anti-mouse IgG and followed by streptavidine peroxidase reagent. Color was developed by adding 3'-amino-9-ethylcarbazole (AEC) in 50 mM acetate buffer pH5.0 and examined under light microscopy.

13.2 FACS analysis

2x10^5 BLCLs were washed 2 times with PBS and resuspended in Cytifix/Cytoperm solution for 20 minutes at 4°C. The permeabilized BLCLs were washed 2 times with 1xPerm/Wash solution (Pharmingen) and stained for NS3 protein by using anti-NS3 (5F-1) for 30 minutes at 4°C. After washing 2 times with the same solution, these cells were stained with rabbit anti-mouse Ig FITC (F0261) for 30 minutes in the dark
at 4 °C. The cells were washed 2 times with the same solution and resuspended in 250 μl staining buffer before FACS analysis. FACS analysis was performed by a FACscan Flow cytometer and analyzed with CellQuest Software.

14. Statistical Analysis

The differences between the prevalence of the T cell responses to individual HCV proteins, for each group of genotypes, were analyzed by $\chi^2$ or Fisher’s exact test, with two degrees of confidence. Values of $p < 0.05$ were considered to be statistically significant.