

## 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 : NKNT-3 (kindly provided by Dr. Bart L. Haagmans)
2. *E.coli* INVØF' (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- $\gamma$  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  $\mu$ l, 200  $\mu$ l and 1000  $\mu$ l (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  $\mu$ M 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. CO<sub>2</sub> humidified incubator (TC2323 Shellab, 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.  $\beta$  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-benzidine (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-indolylphosphate/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. H<sub>2</sub>O<sub>2</sub> (Sigma, USA)
38. Hepes (Merck, USA)
39. NaCl (Sigma, USA)
40. KCl (Sigma, USA)
41. Na<sub>2</sub>HPO<sub>4</sub> (Merck, USA)
42. CaCl<sub>2</sub> (Merck, USA)
43. PBS (Sigma, UK)
44. Na<sub>2</sub>CO<sub>3</sub> (Sigma, UK)
45. NaHCO<sub>3</sub> (Sigma, UK)
46. NaN<sub>3</sub> (Sigma, UK)
47. KH<sub>2</sub>PO<sub>4</sub> (Sigma, UK)
48. MgCl<sub>2</sub> (Sigma, UK)
49. Sodium-N-Lauroyl-sarcosinate (C<sub>15</sub>H<sub>28</sub>NNaO<sub>3</sub>) (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. <sup>3</sup>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- $\gamma$ -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  $-70^{\circ}\text{C}$  for one night before transferring to liquid  $\text{N}_2$  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<sup>(146)</sup>. One hundred  $\mu\text{l}$  of each sample was mixed with 500  $\mu\text{l}$  GTC-2ME (Appendix A). After inverted mix and vortexing, RNA was extracted by the mixture of 50  $\mu\text{l}$  2M sodium acetate, 500  $\mu\text{l}$  phenol, 100  $\mu\text{l}$  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  $4^{\circ}\text{C}$ . The aqueous phase which contain RNA was transferred to a new tube, mixed with 4  $\mu\text{l}$  glycogen and 600  $\mu\text{l}$  isopropanol and then placed in  $-20^{\circ}\text{C}$  freezer for at least 3 hours to precipitate RNA. Sedimentation of RNA was performed by centrifugation at 14000 rpm for 20 minutes at  $4^{\circ}\text{C}$ . 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  $\mu\text{l}$  DEPC (diethylpyrocarbonate) treated sterile water and directly used as a template for RT-PCR. The RNA was heated to  $65^{\circ}\text{C}$  for 5 minutes, cooled on ice and incubated in a reaction mixture containing 1x RT buffer, 0.3  $\mu\text{M}$  primer (410), 0.5 mM dNTPs, 20 U Rnasin<sup>®</sup> ribonuclease inhibitor and 100 U M-MLV reverse transcriptase at  $37^{\circ}\text{C}$  for 1 hour to generate cDNA.

cDNA (5  $\mu\text{l}$ ) was amplified by nested PCR as followed. The nested PCR of core gene was amplified for HCV DNA screening by 2 sets of primers.<sup>(10,13)</sup> 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  $95^{\circ}\text{C}$  for 3 minutes, denaturation at  $94^{\circ}\text{C}$  for 1 minutes, annealing at  $48^{\circ}\text{C}$  for 2 minutes, extension at  $72^{\circ}\text{C}$  for 2 minutes, concluded by a final extension step at  $72^{\circ}\text{C}$  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 4** Primer sequences for HCV RNA detection

Primer	Length	sequences (5'→3')	position	usage	amplified gene
410	20	ATGTACCCCATGAGGTCGGC	409-390	outer reverse	core
954	25	ACTCCCTGATAGGGTTGCTTGCGAG	-54-(-31)	outer forward	core
951	21	CACTGTRAGGGTATCGATGAC	383-364	inner reverse	core
953	24	AGGTCTCGTAGACCGTGCATCATG	-21-(-3)	inner forward	core
NNRdF	24	CAGTATGGATCTGGCCGTG GCTGT	2896-2912	forward	NS3
NNRdR	20	AGGGTGGGCTTGAGGCGAAT	4853-4834	reverse	NS3

## 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 *Bst*N I were used as described by Buoro et al<sup>(14)</sup>. The second one, a method described by Mellor et al<sup>(13)</sup>, 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 volumn 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.

Type on 405 bp	1a	1b	1c	2a	2b	2c	3a	3b	4a	5a	6a
<i>Acc I</i>	202	394	394	281	394	281	281	394	219	394	394
	200	8	8	113	8	113	113	8	175	8	8
	8			8		8	8		8		
<i>Mbo I</i>	296	296	296	181	142	181	296	180	244	296	181
	106	106	106	115	106	115	106	106	106	106	106
		or		106	77	106		77	52		77
		236			39			39			38
		109			38						
<i>BstN I</i>		144	243	144		243				243	
		158	159	138		148				159	
		99		99		11					
				11+1							

**Table 6** Electropherotype patterns from *Ava* I and *Sma* I digestion of the 405 bp core fragment

Pattern	Fragment length (bp)				
A1*	148	101	94	38	24
A2	148	101	94	62	
A3	148	139	94	24	
A4	172	139	94		
A5	139	94	84	64	24
A6	233	148	24		
A7	148	94	87	52	24
A8	172	94	87	52	
A9	172	101	94	38	
A10	163	148	94		
A11	260	94	51		
A12	311	94			
A13	210	101	94		
S1*	163	148	94		
S2	257	148			
S3	259	94	52		
S4	148	111	94	52	
S5	311	94			

\* A=*Ava* I , S=*Sma* I

Interpretation :

Genotype	common patterns
1a	A1S1, A2S1
1b	A3S1, A4S5
6a	A12S5

## 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  $1 \text{ OD } 260 = 50 \mu\text{g double-stranded DNA}$ . Between 10 and 30 ng/ $\mu\text{l}$  (3-6  $\mu\text{l}$ ) of each DNA sample were subjected to cycle sequencing using 8  $\mu\text{l}$  of dye terminator from a DNA sequencing kit and 3.2 pmole of specific primer (in a final reaction volume of 20  $\mu\text{l}$ ) 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.

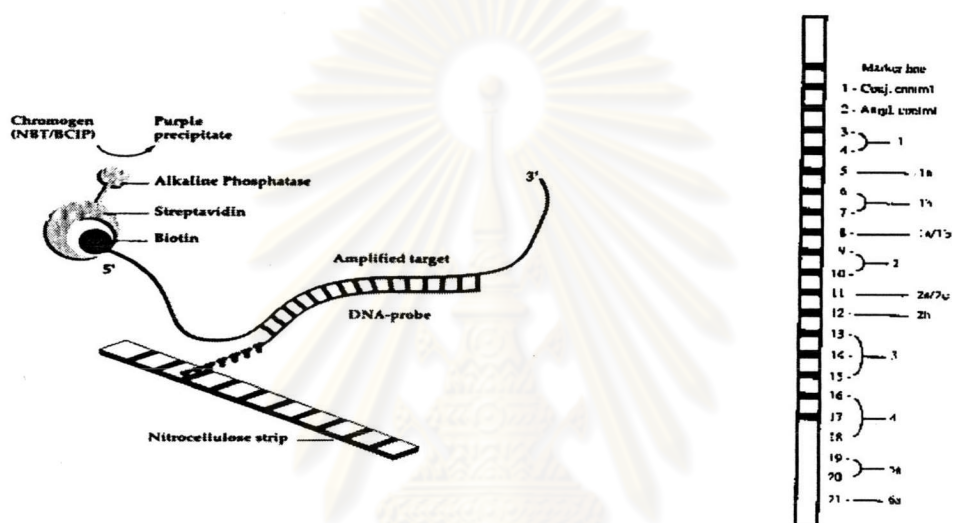


Figure 5 Principle of INNO-LiPA method (left) and the strip for result interpretation (right)

### 6. HCV-specific lymphocyte proliferation assays

Proliferation assays were performed as follows. PBMCs ( $2 \times 10^5$  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  $\mu\text{g/ml}$  final concentrations. After 6 days, 1  $\mu\text{Ci}$  of  $^3\text{H}$ -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  $\geq 3$  and  $\Delta$  cpm (the difference between

cpm obtained in presence of antigen and cpm obtained in the absence of antigen) was > 2000.

## 7. Detection of IFN- $\gamma$ production

Two methods were used for detection of IFN- $\gamma$  in this study.

### 7.1 ELISPOT method

Nitrocellulose-bottom Silent Screen Plate 96-well (Nalge Nunc International) were coated with 100  $\mu$ l of the IFN- $\gamma$  monoclonal antibody at the concentration of 1  $\mu$ g/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  $\mu$ l of R10F containing  $1 \times 10^5$  T cells or  $2 \times 10^5$  PBMCs together with indicated stimuli, or a positive control, PHA(1 $\mu$ g/ml). The mixtures were incubated for 24 hours at 37°C in humidified CO<sub>2</sub> incubator.

After incubation, the cells were removed by washing the plate 6 times with PBS containing 0.05% Tween20. 100  $\mu$ l of the biotin-conjugated anti-IFN- $\gamma$  was added to each well at the concentration of 1  $\mu$ g/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  $\mu$ l of streptavidin-alkaline phosphatase (Mabtech AB) for 1 hour. Unbound conjugate was removed by washing thoroughly with PBS, and finally, 100  $\mu$ l 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- $\gamma$  production by PBMCs induced by HCV proteins,  $2 \times 10^5$  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- $\gamma$  for overnight at 4 °C and the assay was further performed as described above.

For detection of IFN- $\gamma$  production induced by BLCLs that present HCV proteins,  $1 \times 10^5$  T cells or  $2 \times 10^5$  PBMCs were incubated with  $2 \times 10^4$  inactivated BLCLs presenting HCV proteins (or medium with PHA as positive control) in a round bottom 96 well plate in  $37^\circ\text{C}$  incubator for 4 hours before transferring to anti-IFN- $\gamma$  -coated plate. Detection of anti-IFN- $\gamma$  production was continued as described above.

#### 7.2 Intracellular IFN- $\gamma$ detection using FACS analysis

Liver-derived T cell lines ( $2 \times 10^6$  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  $\mu\text{l}$  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  $\mu\text{l}$  staining buffer and then divided into 3 tubes. Cells in each tube were then stained for T cell surface markers and intracellular IFN- $\gamma$  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- $\gamma$  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  $4^\circ\text{C}$ . The permeabilized cells were washed 2 times with 1x Perm/Wash solution (Pharmingen) and stained for intracellular IFN- $\gamma$  production by using anti-IFN- $\gamma$ -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  $\mu\text{l}$  staining buffer before FACS analysis. FACS analysis was performed by FACScan 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 out-patients 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

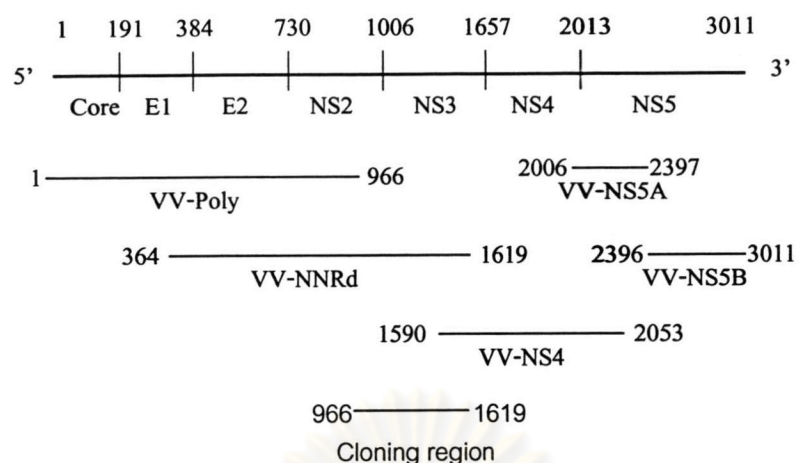
*Dnase I* for 30 minutes in 37 °C incubator. The suspension was filtrated through a cell strainer (Becton Dickinson) and centrifuged at 1,600 rpm for 10 minutes. After discharging the supernatant, the pellet was resuspended and cultured in tissue culture medium (R10F) in the presence of irradiated allogeneic 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,  $1 \times 10^6$  PBMC cells were placed into a 50 ml conical tube. The culture supernatant containing EBV was added, incubated for 2 hours in 37°C humidified CO<sub>2</sub> 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 cm<sup>3</sup> tissue culture flask containing 5 ml fresh R10F and further incubated for 1-2 weeks in humidified CO<sub>2</sub> 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 NNRd region when a biopsy was taken and tested. We decided to use LILs of 816 and antigen in NNRd 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 rVV-HCV carefully, NNRd 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.



**Figure 6** Schematic diagram of all recombinant vaccinia-HCV (rVV-HCV) used in this study. The cloning region (aa 966-1619) was shown in the bottom and covered the NS3 gene.

#### 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* INV $\alpha$ F'. The clones were selected by X-gal/IPTG and ampicillin resistance. Recombinant plasmid was purified and inserted DNA was cut with the restriction enzyme *EcoR* I followed by a fill-in and dephosphorylation step. DNA was subcloned into a VR1012 Neo<sup>+</sup> mammalian expression vector (VRNeo<sup>+</sup>, modified from VR1012, Vical by Noppornpanth S.) at the *EcoR* V site and pNS EBV-based vector at the *BamH* I site and transformed into *E. coli* INV $\alpha$ F'. 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  $5 \times 10^5$  cells per well. Culture was kept at  $37^\circ\text{C}$  in humidified  $\text{CO}_2$  incubator for 24 hours. Twenty micrograms of purified vectors were transfected into these cells using Lipofectamine 2000 (LF2000) reagent according to the manufacturers' protocols.

$7 \times 10^6$  BLCLs were resuspended in  $200 \mu\text{l}$  K-PBS buffer (Appendix A)<sup>(136,147)</sup> Purified pNS and pNS/N16 vectors were also resuspended in  $100 \mu\text{l}$  K-PBS buffer and mixed individually to each tube of cells. The cell mixture was transferred to electroporation cuvette and pulsed at  $390 \text{ V}/500 \mu\text{F}^{(136)}$  in Gene Pulser apparatus. After the electroporation, cells were chilled on ice for 10 minutes and transferred to  $25 \text{ cm}^3$  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%  $\text{H}_2\text{O}_2$  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  $4^\circ\text{C}$  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

$2 \times 10^5$  BLCLs were washed 2 times with PBS and resuspended in Cytifix/Cytoperm solution for 20 minutes at  $4^\circ\text{C}$ . 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  $4^\circ\text{C}$ . 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.



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