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# Target HCV NS3 CD4$^+$ Th1 epitope to major histocompatibility complex class II pathway

Ming Gao[1,2], Hai-Ping Wang[1,*], Yan-Ning Wang[2], Yong Zhou[1] & Quan-Li Wang[1]
[1]*Lab of Blood-borne Virus, Institute of Transfusion Medicine, 27(9) Taiping Road, 100850, Beijing, China*
[2]*LanZhou Jincheng Hospital, 98 west street XiaoXiHu, 730050, QiLiHe, LanZhou, Gansu, China*
**Author for correspondence (Fax: +8610-68151876; E-mail: wanghp119@hotmail.com)*

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**Abstract**

A hepatitis C virus (HCV) plasmid vaccine was constructed, based on class II-associated invariant chain peptide (CLIP) substitution which endogenously targets HCV non-structure protein 3 (NS3) CD4$^+$ T helper 1(Th1) epitope (1248AA-1261AA) to major histocompatibility complex (MHC) class II antigen. The *in vitro* expression results demonstrated that the vaccine was expressed efficiently in COS-7 cell line. The expressed protein could co-localize in endo-membrane system with BALB/c mouse MHC class II molecule I-A$^d$. The recombinant invariant chain molecule could aggregate with BALB/c mouse I-A$^d$ molecule and form the theoretical nonomer structure in the COS-7 cell line. The assembled molecules migrate to the cell surface by exocytosis. This has implications for HCV vaccine development.

## Introduction

About 170 million people are infected with Hepatitis C virus (HCV) worldwide, and 55–85% of acute Hepatitis C virus HCV infections result in chronic hepatitis, which may lead to cirrhosis and hepatocellular carcinoma (Hoofnagle 1997). Currently, the most effective treatment for chronic HCV infection is a combination therapy with interferon-$\alpha$ and ribavirin but, across all genotypes, only 50%of treated patients have sustained benefit from antiviral therapy (Poynard *et al.* 1998, McHutchison *et al.* 1998), so a preventive or therapeutic vaccine is very desirable. The CD4$^+$ T helper 1 (Th1) cell response to the nonstructural protein 3 (NS3) is present in acute infection patients who clear the virus, whereas those who progress to chronic infection lack this response (Diepolder *et al.* 1995, Missale *et al.* 1996, Tsai *et al.* 1997). In established chronic HCV infections, CD4$^+$ T cell responses to the NS3 protein are almost totally absent, whereas antiviral therapy appears to activate these responses (Zhang *et al.* 1997b, Cramp *et al.* 2000).

Peptides endogenously synthesized by gene introduction cannot readily enter the major histocompatibility complex (MHC) class II antigen presentation pathway. Here, by substitution of class II-associated invariant chain peptide (CLIP) with HCV NS3 CD4$^+$ Th1 epitope, we have utilized the invariant chain (Ii),which is an accessory protein involved in the duration of MHC class II maturation and peptide loading (Busch *et cal.* 2000), to target endogenously synthesized HCV epitope to the antigenic-peptide-binding groove of MHC class II $\alpha\beta$ dimers. Our results demonstrated that invariant chain recombinant could express efficiently and assemble with BALB/c mice I-A$^d$ molecule in the COS-7 cell line. The assembled molecules migrate to the cell surface maybe through exocytosis. This may have some implications for HCV therapeutic vaccine development.

## Materials and methods

### *Construction of eukaryotic expression vectors*

The genes encoding BALB/c mice MHC class II molecule I-$A^d$ were cloned using RT-PCR. Total RNA was extracted from mouse spleen cell using Trizol RNA extraction reagent (Invitrogen Life Technologies). Thereafter, the first-strand cDNA was synthesized by SuperScript II reverse transcriptase (Invitrogen Life Technologies) using Oligo$(dT)_{12–18}$ primer following the manufacturer's instruction. The PCR reaction was carried out by using specific primers for the $\alpha$ and $\beta$ chain (Table 1). The purified PCR products were inserted into pGEM-T vector (Promega, USA) for sequencing. The genes, which code for $\alpha$ and $\beta$ chain, were cloned into pIRES bicistronic vector (Clontech Laboratories, Inc.) sequentially and then subcloned into pDs-Red1-N1 vector (Clontech Laboratories, Inc.).

The invariant chain was amplified by PCR using primers mIi1 and mIi2 (Table 1). The PCR product was sequenced as mentioned above. The identified invariant chain was inserted into pCI-neo eukaryotic expression vector (Promega, USA). Invariant chain recombinant was obtained by three PCR amplifications. The first amplification used primers mIi1 and mIi-NS3-1, the second mIi2 and mIi-NS3-2, and the last one mIi1 and mIi2. The purified final PCR product was inserted into pGEM-T vector for sequencing identification and then was cloned into pCI-neo expression vector. Then another PCR amplification was carried out and the amplified invariant hybrid was inserted into pEGFP-N1 vector using the *Eco*RI and *Bam*HI restriction enzyme site.

### *Cell culture*

NIH3T3 and COS-7 cell line were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), 2 mM glutamine and 10 mM HEPES, at 37 °C with 5% $CO_2$ atmosphere.

### *Transient transfection of NIH3T3 and COS-7 cell line*

Transfection was carried out using Lipofectamine2000 (Invitrogen Life Technologies) according to the manufacturer's recommendation. Briefly, 3 $\mu$g plasmids and 5 $\mu$l liposome were mixed thoroughly in serum free DMEM, incubated for 20 min at room temperature. Cells which were seeded in a 6-well culture plate the day before were washed three times with serum free DMEM. Then the prepared mixture was added and an additional 900 $\mu$l DMEM was supplied. The transfected cells were incubated for a further 6 h. At the end of transfection, 10% (v/v) FBS was added.

### *RT-PCR for detection of foreign DNA transcription*

To detect the transcription of foreign DNA, RT-PCR was performed using a set of strand-specific primers 1, 2, 3 and 4 respectively (Table 1). The total RNA preparation was described above. The first-strand cDNA was synthesized by AMV reverse transcriptase (Promega) using specific primers following the manufacturer's instructions.

*Table 1.* PCR primers used in plasmid construction.

1. H-2 I-A $\alpha$ chain up stream *Nhe*I: 5′CTAGCTAGCATGCCGTGCAGCAGAGCTCTGAT3′
2. H-2 I-A $\alpha$ chain down stream *Eco*RI: 5′CCGGAATTCTCATAAAGGCCCTGGGTGTCTGG3′
3. H-2 I-A $\beta$ chain up stream *Xba*I: 5′TGCTCTAGA ATGGCTCTGCAGATCCCCAGCCT3′
4. H-2 I-A $\beta$ chain down stream *Not*I: 5′ATAAGAATGCGGCCGCTCACTGCAGGAGCCCTGCTGG3′
5. mIi1 *Eco*RI: 5′CCGGAATTCATGGATGACCAACGCGACCTCAT3′
6. mIi-NS3-1(48mer): 5′CAAGACAAGTACCTTGTACCCAAGCTTCATGCGAAGGCTCTCGAGTTG3′
7. mIi2 *Xba*I: 5′GCTCTAGATCACAGGGTGACTTGACCCAG3′
8. mIi-NS3-2(53mer):5′AAGGTACTTGTCTTGAACCCGTCTGTTGCCGCCACTATGGATAACATGCTCCT3′

## Results

*Construction of HCV minigene DNA vaccine*

HCV minigene DNA vaccines pCI-Ii/HCV-NS3-Th1 and pEGFP-Ii/HCV-NS3-Th1 were constructed according to standard molecular cloning methods, as illustrated in Figure 1. The construction of bicistronic plasmid is illustrated in Figure 2. All cloned genes were sequenced and determined correctly before being inserted into eukaryotic expression vectors.

*Expression of different plasmids* in vitro

The NIH3T3 and COS-7 cell lines were grown on cover slips until they reached 50–60% confluence and then were transfected. The RT-PCR results demonstrated that foreign genes were transcribed efficiently *in vitro* (Figure 3).

Fluorescence microscopy of COS-7 cells transfected with pEGFP-Ii/HCV-NS3-Th1 and pRed-I-$A^d$ showed that foreign genes could express even if encoding genes of GFP and RFP fused to the *C*-terminus of target genes, respectively (Figure 4). Although the foreign genes could be expressed efficiently (Figure 4a and Figure 4b), the efficiency of two different plasmids entering the same cell is very low (Figure 4c).

*Localization of expressed foreign proteins in COS-7 cell line*

The invariant chain recombinant with GFP fused to its *C*-terminus could be localized on inner membrane systems, whereas the GFP protein distributed extensively in COS-7 cell cytoplasm as well as karyon. We can conclude that the *N*-terminal signal peptide of invariant chain results in the localization characteristics of fusion protein (Figure 5a, b, respectively). Similarly, the localization characteristics of recombined I-$A^d$ was determined by $\alpha\beta$ chain signal peptide compared with pDs-Red1-N1 pure vector.

*Co-expression and co-localization of foreign proteins in COS-7 cell line*

COS-7 cell line was transfected with pEGFP-Ii/HCV-NS3-Th1 and pRed-I-$A^d$ plasmid simultaneously. Both foreign proteins localized on the inner membrane system (Figure 6a, b and d). Invariant chain recombinant could assemble with I-$A^d$ after being synthesized (Figure 6d, e and f). In our research, we observed the assembled protein migrating to the cell surface through exocytosis (Figure 6g, h and i).

## Discussion

Several groups have demonstrated the efficiency of invariant chain as a vehicle for endogenously targeting antigen epitopes to the MHC class II molecule cleft (van Bergen *et al.* 1997, Fujii *et al.* 1998, Malcherek *et al.* 1998, Sponaas *et al.* 1999). Specific $CD4^+$ T cell responses to HSP60 and OVA have been induced (Esther *et al.* 2001, Nagata *et al.* 2002) but, until now, no research

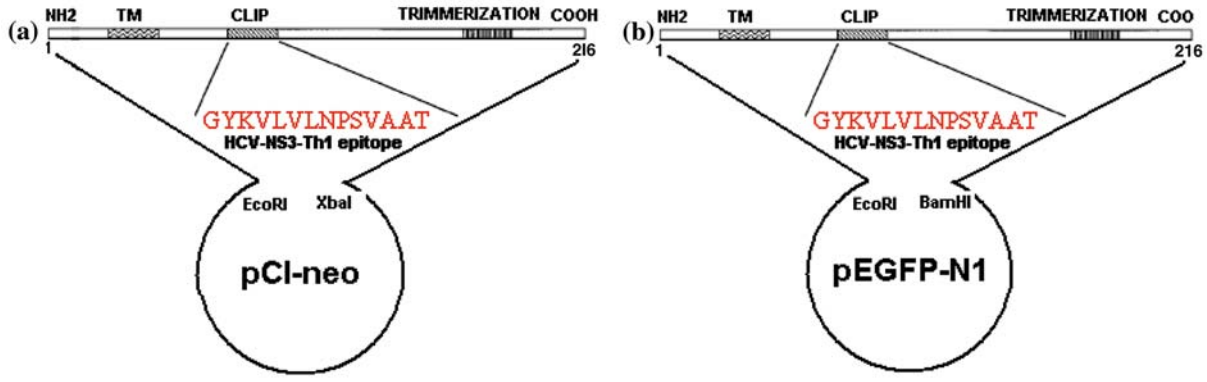


*Fig. 1.* Schematic representation of plasmid construction. (a) The construction of pCI-Ii/HCV-NS3-Th1, (b) The construction of pEGFP- Ii/HCV-NS3-Th1.

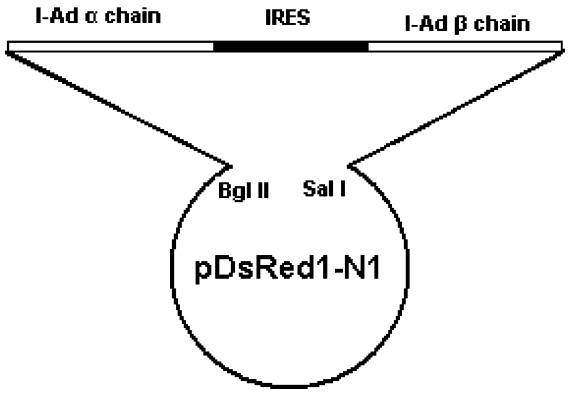


*Fig. 2.* Schematic representation of construction of pRed-I-A$^d$ plasmid.

has reported that the invariant chain molecule could be used as a vehicle of DNA vaccine against an infectious disease. Here, we have constructed an invariant chain recombinant in which CLIP was replaced by HCV NS3 Th1 epitope. GFP and RFP were used as reporter genes for foreign protein subcellular localization. The results demonstrated that the invariant chain recombinant could assemble with mouse MHC class II molecular I-A$^d$ in transfected cells. Co-localization results showed that the two foreign proteins were localized in the inner membrane system simultaneously. Neither targeting characteristic nor localization specialty of the invariant chain hybrid was influenced by substitution of invariant chain CLIP with HCV NS3 Th1 epitope compared with wild type invariant chain (Figure 7).

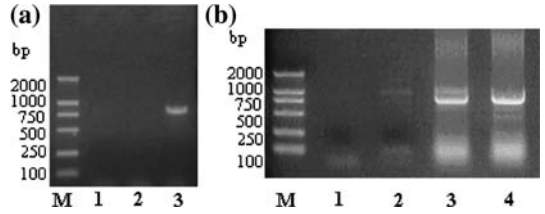


*Fig. 3.* RT-PCR results of transfected COS-7 and NIH3T3 cell line. (a) pCI-Ii/HCV-NS3-Th1 transfected COS-7 cell line. M. DL2000 DNA molecular weight marker, 1. negative control, 2. pCI-neo plasmid transfected COS-7 cell line, 3. pCI-Ii/HCV-NS3-Th1 transfected COS-7 line(814 bp target gene is visible). (b) RT-PCR result of NIH3T3 cell line transfected with pIRES-I-A$^d$. M. DL2000 DNA DNA molecular weight marker, 1, normal cells amplified with I-A$^d\alpha$ chain primers, 2. normal cells amplified with I-A$^d\beta$ chain primers, 3. Transfectants amplified with I-A$^d\alpha$ chain primers (771 bp), 4. Transfectants amplified with I-A$^d\beta$ chain primers (792 bp).

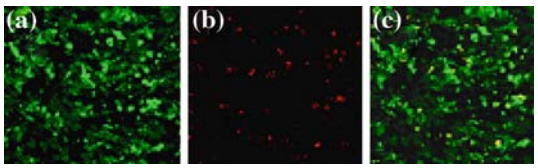


*Fig. 4.* Fluorescence microscopy observation of COS-7 cells transfected with pEGFP-Ii/HCV-NS3-Th1 and pRed-I-Ad. (a) pEGFP-Ii/HCV-NS3-Th1 transfected COS-7 cell line, (b) pRed-I-A$^d$ transfected COS-7 cell line, (c) merge (10×10).

In an antigen-presenting cell, the invariant is degraded progressively by proteases in the late endocytic compartment, and leaves CLIP remaining in the antigen-binding groove. The CLIP remains to associate with MHC II molecules until the antigenic peptide load is finally released by HLA-DM. How the MHCII/peptide complex gets to the cell surface remains unknown. Our results suggest that the MHCII/peptide complex may get to the cell surface by exocytosis (Figure 6 j, k and l).

**In conclusion**, we have constructed an HCV minigene DNA vaccine based on invariant chain CLIP substitution. *In vitro* results demonstrated that the invariant chain recombinant could assemble with mouse MHC class II molecular I-A$^d$ and is localized on the inner membrane sys-

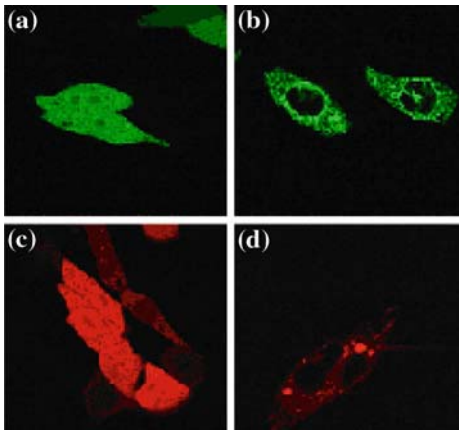


*Fig. 5.* Localization of expressed foreign gene in COS-7 cell line. At 48 h post-transfection, transfected cells grown on glass coverslips in 35-mm culture dishes with special chamber for observation were examined with a Randiance 2100 digital Laser Scanning Confocal Microscope (Bio-Rad, USA). The excitation of GFP was 489 and the emission is 508. The RFP was excited with the 535 wavelength and the emission wavelength is 617. (a) pEGFP-N1, (b) pEGFP-Ii/HCV-NS3-Th1, (c) pDs-Red1-N1, (d) pRed-I-A$^d$ (10×60).

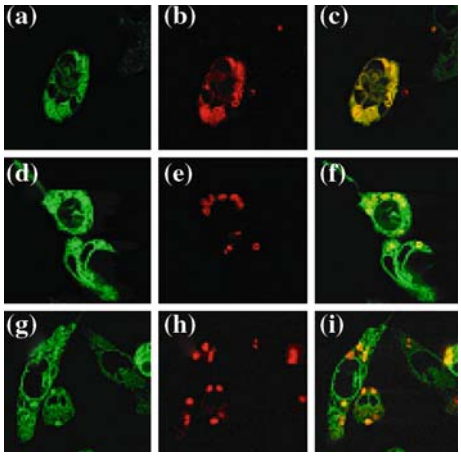


*Fig. 6.* Co-expression and co-localization of foreign proteins in COS-7 cell line. Both mouse major histocompatability complex class II (I-$A^d$) and invariant chain are localized in inner membrane system (a,b,c). Some of them can assemble after synthesis (d,e,f). The assembled I-$A^d$ and invariant chain hybrid, with class II associated invariant chain peptide substituted by HCV NS3 Th1 epitope, can move to cell membrane by exocytosis (g, h, I) (10×60).

tem just like the wild type invariant chain. Though the efficiency of our DNA vaccine to prime humoral and cellular immune response still needs further investigation, our *in vitro* study may have some implications for HCV vaccine development.

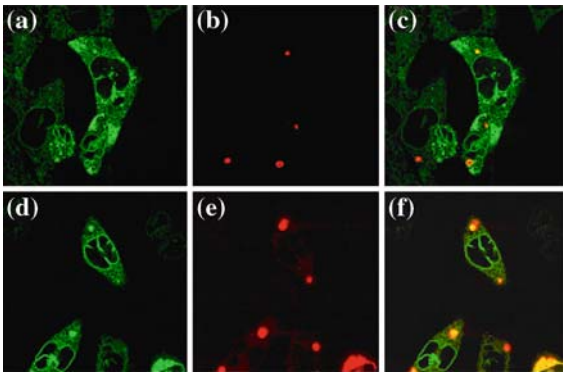


*Fig. 7.* The substitution of class II associated invariant chain with HCV NS3 Th1 epitope did not affect the characteristic of invariant chain. Wild type invariant chain localized in inner membrane system (a) and assembled with mouse MHC class II molecular I-$A^d$ after synthesis (b, c). The invariant chain recombinant also localized in inner membrane system and assembled with mouse MHC class II molecular I-$A^d$ just like the wild type (d, e, f) (10×60).

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