Novel DNA Vaccine Formulations Against Hepatitis C Virus

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Contents

Abstract

No vaccines are available for hepatitis C virus (HCV) which infects over 71 million people worldwide. Current therapeutic options are very expensive and as a consequence, only around 1% of those diagnosed with Hepatitis C receive treatment each year. Induction of neutralizing antibody (NAb) by vaccination will be important for the successful prevention of HCV infection. HCV envelope glycoproteins E1 and E2 are required for virus entry into host cells making these proteins attractive targets to prevent virus infection.

DNA-based vaccines are appealing candidates for novel vaccine development because they are not infectious and are cost-effective to manufacture on a global scale. However, despite being licensed for veterinary use, DNA vaccine have not been highly immunogenic in large animals. Virus-like particles (VLP), on the other hand, which resemble native viruses but are non-infectious because they lack viral genetic materials, have provided highly encouraging results in clinical trials.

In this thesis the immunogenicity of VLPs and the ease of production of plasmid DNA were combined by designing DNA vaccines encoding VLPs consisting of HCV-core, E1 and E2 proteins (which can self-assemble into VLPs following expression). This vaccine also encoded a cytolytic gene perforin (PRF) to cause cell death and ensure the release of the VLPs from vaccine-targeted cells along with damage associated molecular patterns (DAMPs), which act as natural adjuvants. Bicistronic DNA vaccine constructs encoding HCV structural proteins and PRF were successfully generated and validated. Vaccination with the DNA construct encoding CoreE1E2-PRF generated higher adaptive immune responses in mice than vaccination with a construct unable to induce cell death, therefore confirming an adjuvant effect by PRF. However, these responses were weak compared to those reported in the literature.

Antigen oligomerisation has been shown to improve vaccine immunogenicity. To improve the immunogenicity of a DNA vaccine encoding HCV-E1E2, a novel strategy that incorporates E1 and E2 into heptamers by fusion with the oligomerisation domain of a chimeric C4 binding protein (namely IMX3133 or IMX313P) was used. As the adjuvanticity of IMX313 or IMX313P requires efficient secretion of the oligomerised protein, the leader sequence of the tissue plasminogen activator (tPA) was introduced upstream of the E1 or E2 proteins (tE1 or tE2) from which the transmembrane domains were removed. The use of tE1 and tE2 proteins as separate immunogens or as a single tE1tE2 polyprotein when fused to IMX313 or IMX313P was assessed in vaccination studies in Balb/c mice using prime-boost intra-dermal DNA immunisations. Vaccination with the DNA construct encoding tE1/tE2 fused to IMX313 or IMX313P resulted in increased antibody and cell mediated immune (CMI) responses compared

to the same dose of DNA without IMX313 or IMX313P, while fusion of tE1/tE2 to IMX313P resulted in the highest immune responses. DNA prime/E1E2 protein boost or DNA prime/HCV-VLP boost approaches were then used to further improve the immunogenicity of tE1/tE2-IMX313P DNA constructs. Boosting with E1E2 proteins improved overall antibody responses, compared to boosting with HCV-VLPs or plasmid DNA. However, experiments to examine the neutralisation of binding of labelled VLPs showed that the E1E2 or VLP boost did not improve the neutralising potency of these antibodies or the CMI responses.

This thesis demonstrated that expression of heptamerised and secreted tE1/tE2 from DNA vaccine constructs significantly improved the antibody and CMI responses to HCV E1 and E2 proteins. Most importantly, the antibody induced by these constructs possess neutralising properties.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in the submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Abbreviations

ADCC	Antibody-dependent cell-mediated cytotoxicity
APC	Antigen presenting cells
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CMV	Cytomegalovirus
CTLs	Cytotoxic T lymphocytes
DAA	direct acting antivirals
DAMPs	Damage associated molecular patterns
DCs	Dendritic cells
DNA	deoxyribonucleic acid
eGFP	enhanced green fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
EliSpot	Enzyme-linked immunosorbent spot
ER	Endoplasmic reticulum
GFP	green fluorescent protein
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HCV	Hepatitis C virus
HCVcc	HCV cell culture
HCVpp	HCV pseudoparticles
HEK293T	Human embryonic kidney 293T cells
HIV	human immunodeficiency virus
HVR	hypervariable regions
ICS	Intracellular cytokine staining
ID	Intradermal
IFN	Interferon
Ig	immunoglobulin
IL	Interleukin
IM	Intramuscular
LB	Luria broth
LCs	Langerhans cells
LDH	Lactate dehydrogenase
MCS	Multiple cloning site
MHC	Major histocompatibility complex

MLV	Murine leukaemia virus
MW	Molecular weight
NAbs	neutralising antibodies
NHP	Non-human primate
NHS	Normal human serum
NK	Natural killer cells
NMS	Normal mouse serum
NOD	nucleotide-binding oligomerisation domain
NS	Non-structural
ORF	open reading frame
PAMPs	pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PD-1	programmed cell death 1
PHA	Phytohaemagglutinin
PRF	Perforin
PRR	Pattern recognition receptor
RdRp	RNA-dependent RNA polymerase
RIG	Retinoic acid-inducible gene
RNA	Ribonucleic acid
SC	subcutaneous
SOC	standard of care
Th	helper T cells
TLRs	Toll-like receptors
TMD	Transmembrane domain
TNF	Tumour necrosis factor
tPA	Tissue plasminogen activator
UTR	untranslated region
VISA	virus-induced signalling adapter
VLP	virus-like particles

Chapter 1. Literature Review

1.1. HCV transmission and epidemiology

HCV is an enveloped positive sense single-stranded RNA virus that is estimated to infect more than 71 million people (~1%) of the world's population, making HCV the leading cause of liver disease worldwide [9, 10]. In developing countries, HCV infections are largely transmitted by parenteral exposure to contaminated blood or blood products in the form of transfusion, unsafe therapeutic injections and intravenous drug use [11, 12]. After widespread screening for HCV was implemented in the 1990's in economically developed countries, new cases of transfusion- and organ transplant-associated infections were nearly eliminated, and the vast majority of newly acquired infections are now spread through intravenous drug use and/or needle sharing [13]. The risk of transmission from sexual contact is believed to be low [14].

The incidence of HCV infection is difficult to determine as most acute infections are asymptomatic and therefore remain undetected [15]. HCV infection rates worldwide are believed to be underestimated as prevalence data from many geographical areas are unavailable [16]. In addition, these areas have both limited screening of blood donations and limited HCV testing available [13] suggesting that they might have increasing incidences of HCV infection. The prevalence of HCV infection is much higher (up to 15%) in some African and Asian countries compared to the USA (1.7%) and Europe (1.03%) [13, 17, 18]. Egypt for example has a prevalence of up to 20% which is the highest reported prevalence rate for HCV [13, 17].

HCV is genetically diverse and is currently subdivided into seven major genotypes which are at least 30% divergent in nucleotide sequences, and each of these genotypes is subdivided into numerous subtypes [19, 20]. Based on current evidence, these genotypes appear to have evolved separately in different geographical regions despite displaying similar pathology and transmission characteristics [19, 20]. Genotypes 1, 2 and 3 are prevalent in North America, Europe and Asia, with genotype 1 being the most endemic in these regions. Genotype 4, on the other hand, is predominantly found in the Middle East and Africa but has recently been reported in several European countries [21-23]. Genotypes 5, 6, and 7 are rare. The most common genotypes in Australia are genotypes 1a and 1b (54% prevalence) and 3a (37% prevalence) [24, 25]. In addition to their geographical distribution, HCV genotypes also differ in severity of the disease and establishing persistent infection and may respond differently to therapy.

1.2. HCV Pathogenesis

HCV is largely a silent disease as the acute phase remains largely asymptomatic. Although approximately 20% of infected individuals are able to spontaneously clear the virus, the majority of cases develop chronic infection and these individuals are at high risk of acquiring

serious liver damage including steatosis, liver cirrhosis and hepatocellular carcinoma (HCC) [23].

During the first 6 months after infection which is considered the acute phase, most infections remain undiagnosed. The development of non-specific symptoms including decreased appetite, fatigue, malaise, jaundice, anorexia and fever have been reported from patients who acquired infection via needle stick injury or following transfusion [26]. Elevated serum alanine aminotransferase (ALT) levels and viral RNA (usually detected within 1 to 2 weeks following exposure) are common features of acute infection [26, 27].

HCV infection persisting for more than six months is defined as chronic infection [28]. The outcome of infection depends on a number of viral and host factors including the age of the patient at the time of infection, ethnicity, gender, lifestyle, underlying disease, the viral genotype, viral quasispecies diversity and importantly the efficiency of the infected individual's immune response. During the chronic stage patients may remain symptomless but a proportion will eventually develop conditions such as hepatic steatosis, progressive fibrosis, compensated and later decompensated cirrhosis and ultimately HCC [26, 29]. Once cirrhosis is established, the risk of developing HCC is approximately 4% each year. It is believed that hepatocellular damage likely reflects destruction of HCV-infected cells by cytotoxic CD8+T cells, resulting in proliferation of other hepatocytes. However immunosuppressed patients suffer a more severe course of liver disease than immunocompetent patients, possibly due to unrestrained virus replication (caused by immunosuppression) resulting in increased intracellular levels of virus that result in cytotoxicity [10, 26].

1.3. HCV life cycle and molecular biology

The HCV enters host cells via clathrin-mediated endocytosis and pH-dependent release from early endosomes (Fig. 1.1) [30, 31]. The virus mainly infects hepatocytes by binding to various cell-associated receptors. Based on their function these can be divided into attachment receptors or post-attachment receptors. HCV attachment to cells is believed to be initially mediated by the binding of cellular apolipoprotein E (apoE) and phosphatidylserine (PS) incorporated on the viral envelope to heparan sulfate (HS) proteoglycans (HSPGs) (containing syndecans (SDCs)-1/SDC-2 core proteins) and T cell immunoglobulin and mucin domain-containing protein 1 (TIM-1) respectively on the surface of hepatocytes [32, 33].

Post attachment receptors, on the other hand, do not play critical roles in cell attachment but are important for HCV cell entry and uncoating [34, 35]. These receptors include the tetraspanin CD81 [36], the Niemann-Pick C1–like 1 cholesterol absorption receptor (NPC1L1) [37], the

scavenger receptor class B type I (SR-BI) [38] and the tight junction proteins claudin (CLDN) and occludin (OCLN) [31, 39] which interact with the envelope protein 1 (E1) and E2.

CD81 was the first HCV co-receptor described to interact with the envelope protein E2 [7, 36, 40]. Studies using retrovirus pseudotyped with E1/E2 (HCVpp) [41-43] and cell culture derived HCV (HCVcc) made by transfecting full-length HCV RNA into Huh 7 cells [7, 44, 45] have provided valuable information about the interaction between E2 and CD81. These studies have demonstrated that the large extracellular loop (LEL) of CD81 is an important host cell factor for viral entry as it contains several amino acids residues critical for CD81-E2 binding [46-50]. E2 amino acids residues at positions 415, 420, 527, 529, 530, and 535 have been shown to be important for virus particle–CD81 interaction [51, 52].

SR-BI binds to a variety of lipoproteins and is involved in bi-directional cholesterol transport at the cell membrane [38]. The SR-BI extracellular loop has been shown to interact with E2 HVR1 [38] and its amino acids at positions 70–87 are thought to be required for E2 recognition [53].

CLDN1 is expressed in all epithelial tissues but predominantly in the liver [54]. It is localised to the tight junction of hepatocytes and may also be localised to the basolateral surfaces of these cells [55]. Studies have suggested that non-junctional CLDN1 may be involved in HCV entry during post-binding steps [39, 56].

OCLN, a four transmembrane protein expressed in tight junction of polarized cells, is another host cell factor critical for HCV entry [57-59]. OCLN is believed to be one of the HCV host entry factors responsible for the species specificity of HCV as expression of human OCLN has been shown to be required for infection of mouse cells [57]. However, no evidence of a direct interaction with HCV has been reported. Although these receptors confer species specificity, *in vitro* replication of HCV in non-liver cells such as neuroepithelial cells has been described, arguing that HCV might not be strictly hepatotropic [60, 61].

Since it is of positive polarity, the HCV RNA functions directly as mRNA in the cytoplasm of the host cell, where protein translation is initiated through an internal ribosomal entry site in the 5' untranslated region (UTR) (Fig.1.1) [62]. The RNA contains a single open reading frame flanked by two UTRs that encodes a polyprotein which is co- and post-transitionally processed by viral and cellular proteases into structural proteins (core, E1 and E2) and non-structural (NS) proteins (p7, NS2, NS3, NS4A, NS5A and NS5B) (see Fig. 1.2) [63]. Following synthesis and maturation, viral RNA and the NS proteins form membrane-associated replication complexes, which appear as a perinuclear membranous web [64]. These replication complexes can then

catalyse the transcription of negative-sense RNA intermediates from which progeny positive-strand RNA molecules are generated [64]. Capsid proteins and genomic RNA assemble to form a nucleocapsid, which buds through the membranes of the endoplasmic reticulum into cytoplasmic vesicles [10, 65]. Enveloped, mature virions leave the cell via the secretory pathway. However, this final step is still not well understood and is an exciting area for future research.

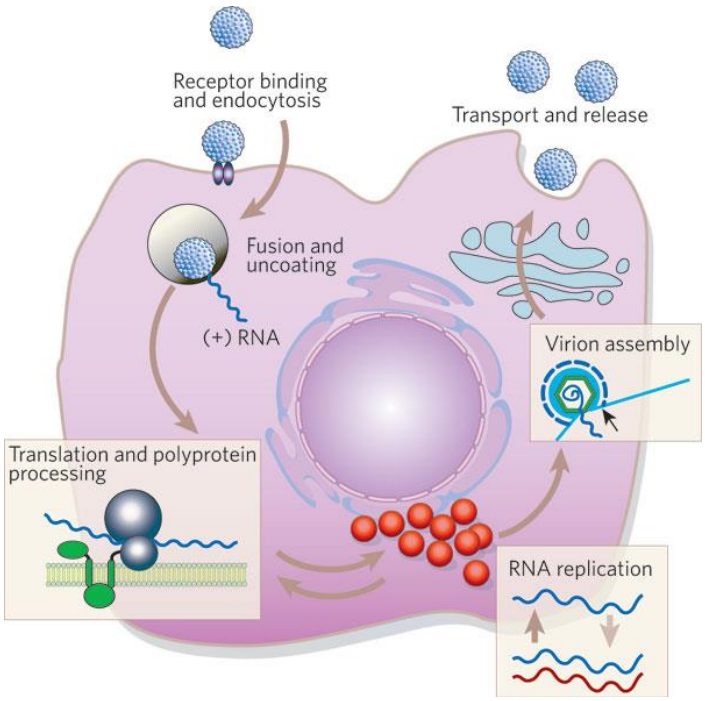

Figure 1.1. The HCV lifecycle. Extracellular virus particles enter hepatocytes by interacting with cell surface receptors, followed by receptor-dependent endocytosis. Upon arrival in early endosomes the HCV glycoproteins mediate fusion of the viral envelope with the endosome and the viral genome is released into the cytoplasm. The viral RNA is then translated to generate the large polyprotein that is processed into 10 mature HCV proteins. Replication complexes formed through the association of the NS proteins with ER-derived membranes replicate the genome. A portion of the newly synthesised positive-sense RNA is packaged into viral capsids in the ER, the resultant nucleocapsid is enveloped by the virus envelope glycoproteins, and the virions are then secreted through the cellular secretory pathway, completing the life cycle (adapted from [7]).

The core protein is the main structural component of the viral nucleocapsid, while the E1 and E2 glycoproteins form a functional heterodimer that mediates viral entry and fusion [66, 67].

The E1 glycoprotein contains a C-terminus transmembrane domain (TMD) with five glycosylation sites, which anchors the ectodomain to the virion (Fig. 1.3). E1 has been shown to be critical for viral entry and the correct assembly and stability of the E1/E2 heterodimer. However, the function of E1 in entry is still unknown [42].

The E2 glycoproteins contains 11 largely conserved N-linked glycosylation sites and 18 conserved cysteines (Fig. 1.3). The E2 receptor binding domain (RBD; residue 384-661), is linked through a conserved C-terminal stem to the TMD and folds independently of other E1/E2 sequences [68]. The binding site for CD81 consists of highly conserved segments within the E2 RBD [36, 51, 68-70] which interact with the LEL of CD81 through Ale182, Asn184, Phe186 and Leu162 on the head subdomain [68, 71].

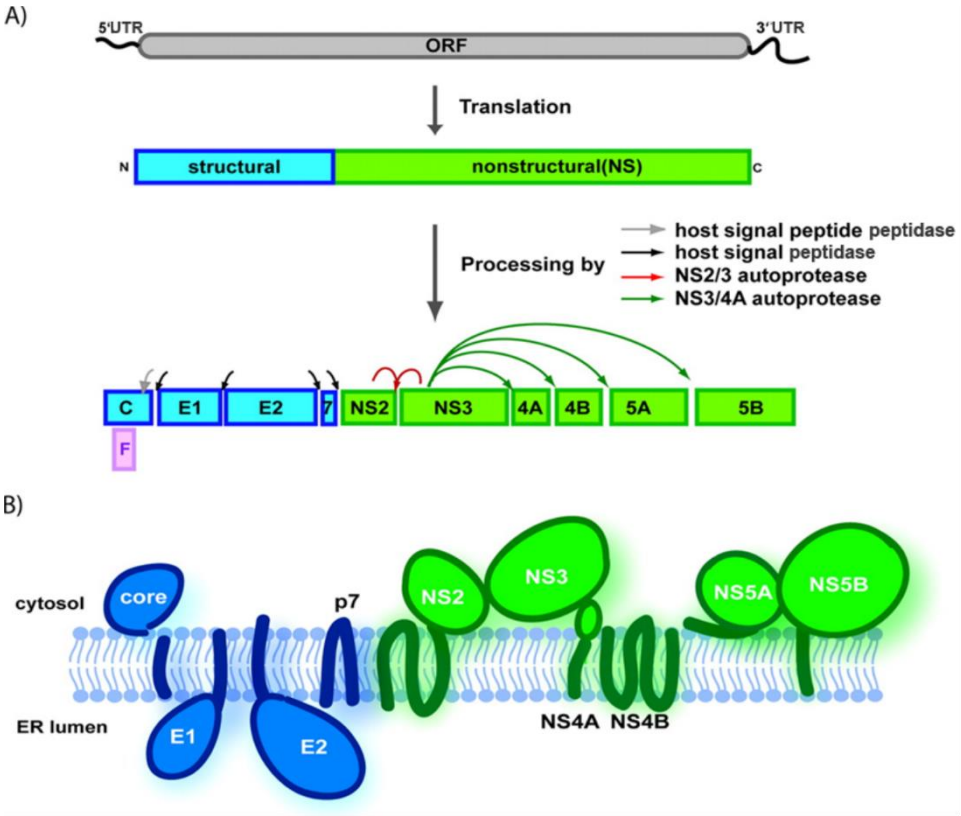

Figure 1.2. The organisation of the HCV genome and protein topology. (A) The HCV genome is a single-stranded RNA encoding a single large open reading frame (ORF) of roughly 3,000 amino acids, flanked by structured 5′ and 3′ UTRs. The translation of the open reading frame, via the activity of an IRES element in the 5′ UTR, generates a large polyprotein that is organized with structural proteins in the amino-terminal third of the polyprotein, followed by the NS proteins required for replication. The polyprotein undergoes a complex co- and post-translational series of cleavage events, catalysed by both host and viral proteases, to produce the 10 individual HCV proteins. (B) The topology of the HCV proteins relative to the ER membrane (adapted from [6, 7]).

Located within the RBD of E2 there are three regions with increased genetic variability characterised as hypervariable regions (HVR) -1 and 2, and the intergenotypic variable region (igVR) (Fig. 1.3). HVR1 is 27 amino acid long [72] and has been attributed to evasion from neutralising antibodies (NAbs) as replication by the error-prone RNA-dependent RNA

polymerase results in high viral quasispecies variability as a consequence [73]. Despite the high sequence variability, the overall basic charge of this region is preserved, probably to maintain interaction with SR-B1 [74] as deletion of HRV1 from the RBD abolishes the interaction with SR-B1 [75]. HRV2 is a region (residue 461-481) downstream of HRV1 flanked by conserved cysteine residues [76]. Another cysteine -flanked variable region igVR (residue 570-580) has been described [77]. This region exhibits a high degree of intergenotypic variation [77]. HRV2 and igVR are believed to be essential to the virion-incorporated E1/E2 structure as deletion of either region is not tolerated and causes E2 to fail to form a heterodimer with E1 resulting in non-infectious HCV particles [78]. No evidence has so far been found to suggest that HRV2 and the igVR are targeted by antibodies and therefore are less likely to be under immune pressure [77].

The hydrophobic p7 protein as well as NS2 are required for assembly and release of infectious particles [79, 80]. The serine protease residing in the amino-terminus of NS3 forms a stable complex with the NS4A cofactor and catalyses polyprotein cleavage at the NS3-4A, NS4A-B, NS4B-5A and NS5A-5B sites (see Fig. 1.2) [81]. NS5A is required for RNA replication and virion morphogenesis [82], while NS5B is a conserved RNA-dependent RNA polymerase (RdRp) required for RNA synthesis [83, 84]

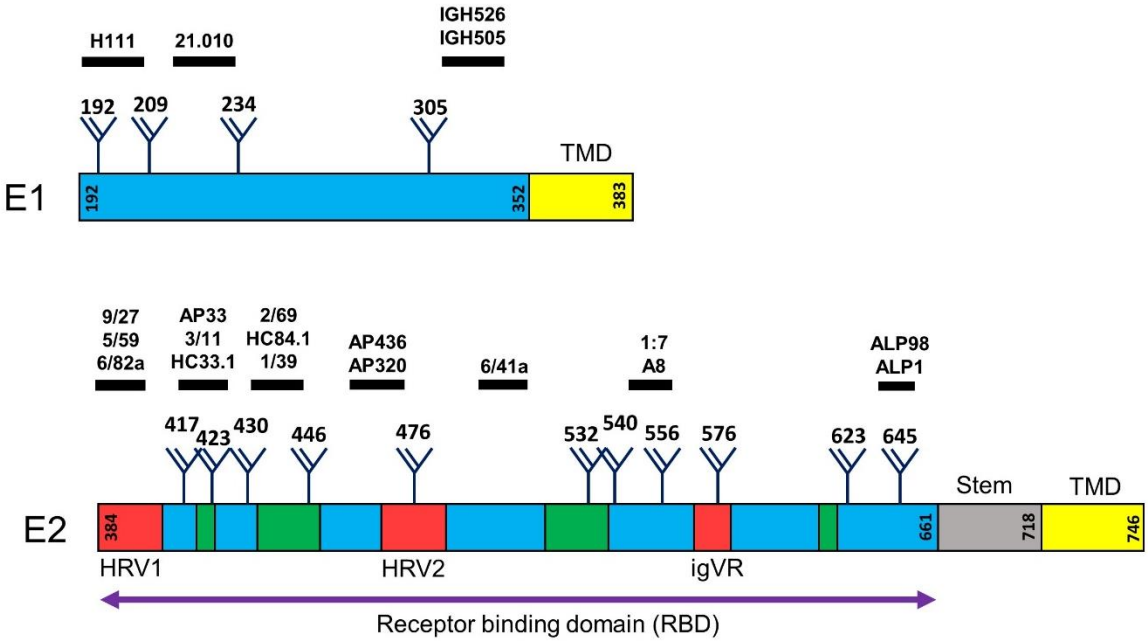

Figure 1.3. Schematic representation of glycoprotein E1 and E2**.** The locations of conserved glycosylation sites are represented with trees and the transmembrane domains are shown in yellow. Regions that are involved in CD81 binding are shown in green. The location of the hypervariable regions in E2 are shown in red and the E2 stem region is indicated in grey. Regions possessing antibody epitopes are shown in black. The alignment of the HCV gene sequence is based on the genotype 1a, H77c (GenBank accession no. AF011751). (Adapted from [4, 5]).

1.4. Immune Responses to HCV Infections

The success of most vaccines depends on the induction of adaptive immune responses, which in the context of viral infections are crucial to either prevent or limit infections. Antigen presenting cells (APC), particularly dendritic cells (DCs), link innate immune responses to adaptive immune responses, and are capable of priming naïve T cells and producing large quantities of cytokines [85, 86]. DCs can capture pathogen proteins and present these to major histocompatibility complex (MHC) restricted helper T (Th) cells in the lymph nodes. With the assistance of CD4+ T cells, B cells secrete antibodies with the potential to neutralize the virus by binding to the proteins on the virus envelope that are necessary for entry into host cells. If the neutralisation is unsuccessful and infection proceeds, then with the help of CD4 T cells, CD8 T cells (also known as cytotoxic T lymphocytes (CTL)) can directly eliminate virus-infected cells by releasing toxins such as perforin and granzyme or antiviral cytokines like interferons (IFN)-γ and tumour necrosis factor (TNF)-α (Fig. 1.4). Perforin (PRF) is a soluble calcium-dependent, pore-forming cytolytic glycoprotein that is crucial for the function of cytotoxic lymphocytes (CTL and natural killer (NK) cells). Once a virus-infected or transformed cell is recognised by CTL, they form an immunological synapse which leads to the fusion of cytotoxic lymphocyte granules (in which perforin is sequestered along with granzyme serine proteases) with the plasma membrane of the CTL, releasing the cytotoxic granules contents into the synapse. In the synapse, perforin binds to the target cell membrane forming transmembrane pores and these allows the diffusion of granzyme into the target cell [87-89]. Once in the target cell, granzymes can then initiate caspase-dependent and caspase-independent apoptotic pathways, which rapidly lead to target cell death [89].

Various innate and adaptive immune components play a crucial role in clearing HCV infection. The innate immune response which includes a network of cells including NK cells, monocytes, macrophages, leukocytes and DCs together with the production of type I interferons and other interferon stimulated genes (ISGs) by hepatocytes present the first line of defence during HCV infection. The innate immune response is activated early during infection and limits progression of infection until the adaptive immune response is activated. The development of antigen-specific adaptive cell mediated immunity [90] has been shown to be crucial for HCV clearance [91]. Vaccines rely on adaptive immune responses to confer long-term specific protection against pathogens of interest. Regardless of the outcome of the infection, the majority of HCV-infected patients develop antibodies against HCV some of which can neutralise viral particles and therefore may have an important function in limiting viral spread [26, 92]. Despite the ability of HCV to evade the host immune responses (see section 1.4.1), ~20% of HCV-infected

patients with acute infection experience spontaneous resolution of viraemia, suggesting that the development of an effective vaccine is likely possible [26, 93].

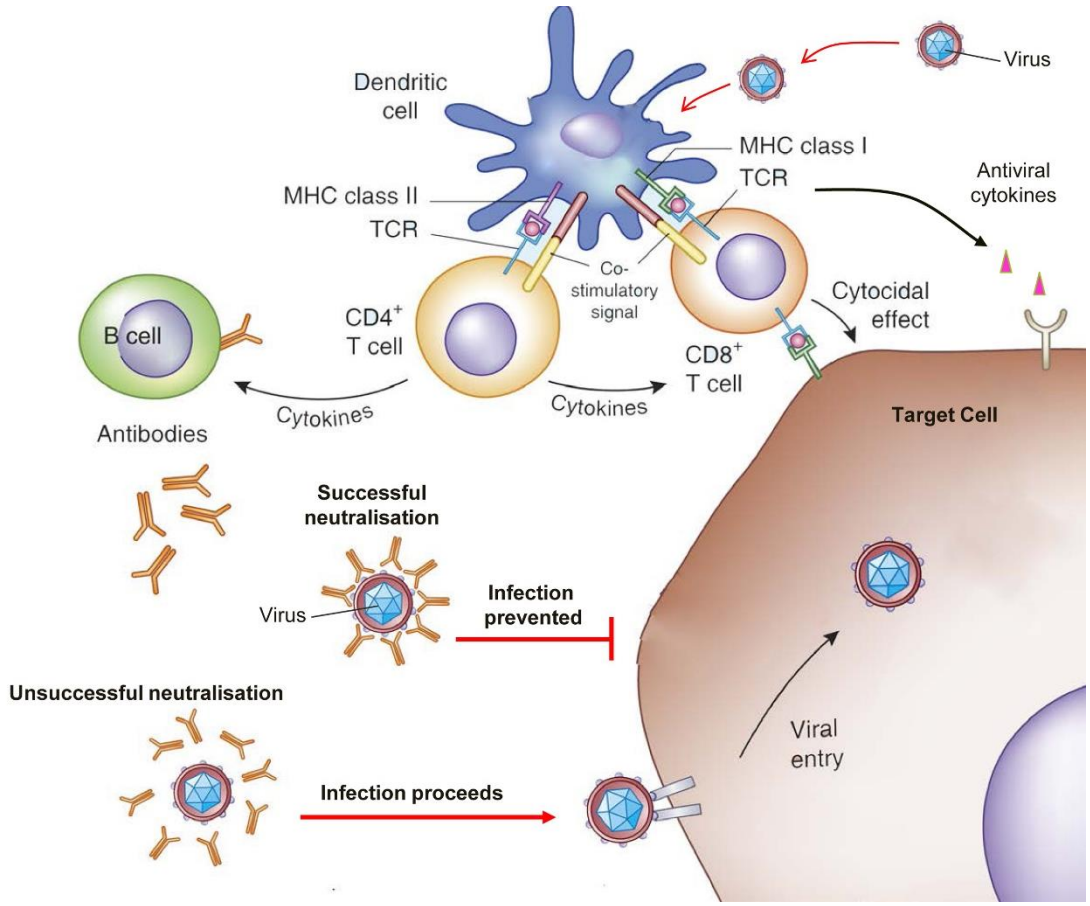

Figure 1.4. Immune responses to viral infections. Humoral immune responses involve B cells that differentiate into antibody secreting plasma cells on encounter with antigen. The cellular immune response involves antigen uptake and processing by DCs and antigen presentation to CD4 and CD8 T cells which drives their differentiation into effector cells (Adapted from [2]).

1.4.1. Innate immunity

Early host control of HCV or other virus infections relies on the innate immune response to inhibit virus spread immediately after infection and is crucial in the development and the shaping of adaptive immunity [94, 95]. To detect invading pathogens, the host immune cells rely on recognition of pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs) found on innate immune cells including NK cells and DCs. Currently, four different classes of PRR families have been identified. These families include transmembrane proteins such as the Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), as well as cytoplasmic proteins such as the Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs) [96-98]. PRRs are a family of transmembrane pattern recognition receptors that

recognise microbial PAMPs and are capable of activating the expression of genes involved in inflammatory and immune responses [96, 99]. Viruses that are not detectable by the endosomal entry pathway can be detected by the cytosolic pathway triggered by binding of viral RNA to the RIG-1 and melanoma differentiation antigen 5 (MDA5) [100, 101]. These viral sensors in turn rely on downstream adaptor proteins to promote the antiviral signalling program. Antiviral type I IFNs (comprising several IFN-α and one IFN-β) and type III IFNs (IFN-λ1, - λ2, and λ3; also designated IL-29, IL-28A, and IL-28B), as well as other proinflammatory cytokines can be triggered as a downstream effect of this signalling, depending on the incoming virus and activation pathway.

During HCV infection, HCV-infected liver cells trigger the earliest host responses. Type I and III IFNs from both HCV-infected hepatocytes and immature DCs [94], are the main cytokine responsible for the induction of an intracellular antiviral state, and for the activation and regulation of the cellular components of innate immunity such as NK cells [102]. Once produced, IFN-I is secreted from the cell and acts in an autocrine or paracrine fashion to promote an antiviral state in infected and neighbouring hepatocytes [102]. IFN-β produced by infected hepatocytes is initiated by TLR3 and RIG-I which recruits the adaptor molecules IFN-β promoter stimulator protein 1 (IPS-1; also referred to as CARD adaptor inducing IFN-β (CARDIF), virus-induced signalling adapter (VISA), and mitochondrial antiviral signalling protein (MAVS)) and Toll-IL-1 receptor domain containing adaptor inducing IFN-β (TRIF). These processes result in downstream signalling, nuclear translocation of IFN regulatory factor (IRF3), and synthesis of IFN-β [103]. Type II IFN (IFN-γ) on the other hand is produced by NK and NKT cells as part of the innate immune system, and by antigen-specific T cells CD4+ and CD8+ T lymphocytes. Type I IFNs are believed to bind to a heterodimer receptor consisting of the interferon-α/β receptor (IFNAR)1 and IFNAR2 [104], while type III IFNs bind a heterodimer receptor consisting of IL10-R2 (also known as IL10RB) and IL28RA. These genes, collectively facilitate the clearance of virus from infected cells and protect uninfected neighbouring cells.

Despite the activation of innate immune response, evidence suggest that HCV has developed several strategies to overcome these host responses at multiple levels. The viral NS3/4A protease cleaves important components of the type I IFN activation cascade, such as TRIF [105] and IPS-1 (MAVS) [106], blocking the activation of several interferon-stimulated genes and thereby preventing TLR3 and RIG-1 signalling [107]. Furthermore, the core protein interferes with Janus kinase/signal transducer and activator of transcription (JAK/STAT) signalling and impairs interferon-stimulated gene (ISG) expression [95, 108]. The NS4B protein induces alterations of the membranous web and suppresses RIG-I mediated IFN-I signalling by binding

to Stimulator of Interferon Genes (STING) [79, 80]. Another key player in HCV-associated immune system attenuation is HCV NS5A which inhibits the ISGs 2'-5' oligoadenylate synthetase (2'-5' OAS) and induces interleukin (IL)-8 which inhibits overall ISG expression [109]. NS5A forms heterodimers with protein kinase R (PKR) and also inhibits cyclophilin A activity thereby blocking IFN effector function [110, 111], while E2 acts as decoy target to PKR [112]. As cytokines which are normally produced during the innate immune response trigger the activation of adaptive immunity, the attenuation of innate immune signalling profoundly affects the subsequent activation of the HCV-specific adaptive immune response.

As noted above, DCs link innate immune responses to adaptive immune responses. These cells process and display antigenic peptides in a complex with MHC on their surface and activate T cells. If infected by viruses, DCs have the ability to process the viral antigens and efficiently present them to T cells, thereby activating these cells. This process in known as direct presentation [3]. Many viruses, however, infect only one or a few cell types and could therefore potentially avoid recognition by not infecting DCs. To overcome this, DCs use cross-presentation, whereby they acquire antigens from extracellular fluids through the process of endocytosis or phagocytosis followed by MHC-class I-restricted presentation to CD8+ T cells (Fig. 1.5).

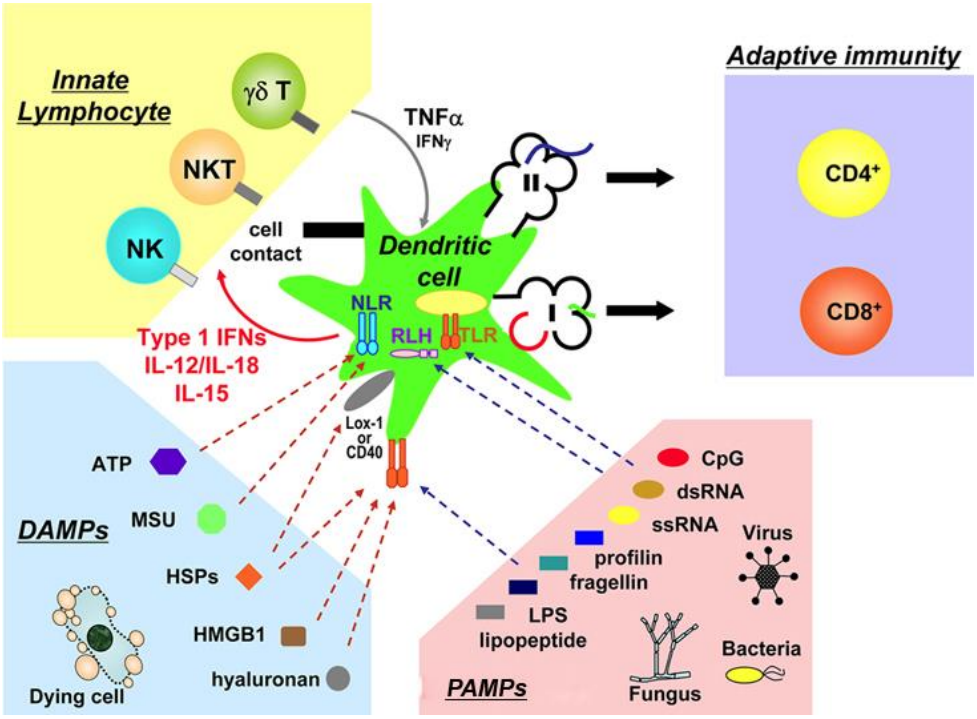

Figure 1.5. Central role of DC in the activation of immune response. DCs respond to three known groups of maturation stimuli including damage associated molecular patterns (DAMPs), pathogen associated molecular patterns (PAMPs), as well as other innate lymphocytes, such as NK cells, NKT cells and γδ T cells through cell-cell contact and cytokines. Once activated DCs can then interact with CD4 and CD8 T cells to drive their antigen-specific activation and proliferation (Adapted from [3]).

A successful vaccine must target DCs and/or otherwise direct the immunogen to DCs for cross presentation [113]. Vaccination with HCV antigen-loaded DC may constitute an efficient and important antiviral therapy for HCV. Several approaches involving DC-based vaccines have been developed and these strategies been shown to effectively induce HCV-specific CD4+ T-cell response as well cytotoxic lymphocytes (CTL) in mice [114-116] and in a phase I human clinical trial [117], suggesting that this might be a successful approach for the priming or boosting of anti-HCV CD8+ T cell responses to aid in the clearance of the virus [117, 118]. Nonetheless during HCV infection, the maturation and functional differentiation of DCs are altered leading to insufficient T cell priming and delayed HCV-specific T cell response [119, 120]. Similarly, the frequency of plasmacytoid DCs (pDCs) as well as their ability to produce IFN-α upon stimulation are impaired in chronic HCV patients [121, 122]. It is possible that HCV proteins play a role in suppressing protective immunity through interaction with DCs as well as other immune cells, however the mechanisms whereby HCV affect DC function remain elusive [123].

NK cells are another type of innate effector cell that frequently reside in the liver and can rapidly exert cytotoxicity and release cytokines. While NK cells potentially contribute to the initial control of HCV infection, there is little evidence that this innate and adaptive immunity can be exploited for vaccine-induced immunity against subsequent viral infection [124]. HCV E2 protein is believed to inhibit NK cell function directly by crosslinking CD81 [125, 126]. However it has also been reported that HCV E2 does not efficiently crosslink CD81 on NK cells when it is a component of infectious virions and NK cell function remained intact after *in vitro* exposure to HCV-antibody complexes [127].

Collectively, these data suggest that HCV interacts with and affects the function of different cells of the innate immune response. This interference differs with regard to cellular levels, targets and outcomes, and results in a deficient adaptive immune response and loss of pathogen elimination function. Nevertheless, the fact that some individuals are able to clear the infection suggest that the cytokine milieu produced by DCs along with the efficacy of costimulatory pathways between DCs and T-cells are important in priming a strong antigen-specific adaptive response, capable of clearing HCV infection [128]. Thus, raising the prospect of targeting this arm of immune response in vaccine development.

1.4.2. Adaptive immunity

Unlike innate immunity, the adaptive immune response is specific and can take weeks to develop following infection. The main components of the adaptive immune response include

the cell mediated immune response (comprised of T lymphocytes) and the humoral immune response (comprised of B lymphocytes).

1.4.2.1. Humoral immunity

Humoral immunity against HCV is characterized by B cell-mediated production of NAbs. NAbs have the ability to prevent viruses and intracellular bacteria from entering host cells by binding to the proteins on the pathogen surface that are necessary for entry into host cells. Although the effectiveness of all current licensed viral vaccines relies on the production of NAb, these antibodies were initially thought to play minor roles in the outcome of HCV infection [129-131]. Their role in viral clearance and disease progression is not well defined, largely due to the lack of assays to measure and quantify their activity [132]. Recent clinical and experimental data have highlighted the importance of NAbs and their contribution to both HCV clearance and protection from reinfection [133-135]. Broadly specific NAb elicited early during infection were shown to correlate with viral clearance [134-136] while individuals who failed to make NAbs progressed to chronic infection. NAbs were detected in a majority of reinfected individuals who managed to clear the virus but was not detected in those HCV-infected patients who progressed to chronic infection [129].

The frequency of B cells which secrete immunoglobulin (Ig) G as well as the overall concentration of IgG in blood are increased during chronic hepatitis C [137]. A small subset of HCV-specific antibodies, usually detectable between 8-20 weeks after infection, can prevent virus binding, entry, or post-entry steps of the viral lifecycle, and therefore prevent viral infection and spread to uninfected cells [131, 132, 137]. However, this response is often delayed during infection, possibly due to a defect in the priming mechanism of B and T cells [132, 137].

A study using homologous viral assays associated acute-resolving HCV infection with early development of NAbs [134]. Moreover, cross-reactive NAbs have been detected in the sera of HCV-infected patients who subsequently cleared the virus after re-exposure, suggesting that NAbs might also be important in protection from re-infection [138]. NAbs have been shown to have a protective role in the chimpanzee animal model of HCV infection [139], however evidence of a similar protective role in patients with acute-resolving infection was not confirmed by some researchers despite the detection of NAbs [132, 140]. Nevertheless, HCV can be cleared in some infected individuals in the absence of NAbs, even in immunocompromised (e.g., hypogammaglobulinemic) patients [140, 141] suggesting the involvement of adaptive cellular immunity.

Although antibodies produced during HCV infection target epitopes within both structural and NS proteins, NAb target epitopes within E1 and E2 proteins, or the E1E2 heterodimer.

Glycoproteins E1 and E2 form a heterodimer that mediate viral entry and NAbs AR4 and AR5 have been shown to recognise conformational epitopes on the E1E2 heterodimer with broad neutralising cross-reactivity between diverse HCV genotypes [142].

Few NAb that specifically target E1 have so far been identified [4, 143]. However, It is still unclear whether this is due to the poor immunogenicity of E1 as E1-specific antibodies have been detected only at low levels in HCV patients [134, 144]. Despite their low titres *in vivo*, E1-specific antibodies have been reported to contribute largely to the overall neutralisation of HCVpp *in vitro* [145]. Moreover, anti-E1 antibodies have been found to be difficult to induce with vaccines expressing the E1E2 heterodimer but are more efficiently induced following separation of E1 from E2. This suggests that E2 is more immunogenic or that E1 neutralising epitopes are masked by E2 [146, 147]. Two monoclonal antibodies, IGH505 and IGH526 (Fig. 1.3), isolated from a patient who cleared a genotype 1b infection, were found to recognise overlapping but distinct epitopes within E1 residues 307-340 and neutralise genotype 1a, 1b, 4a, 5a, and 6a viruses [148]. Additional neutralising epitopes within E1 have been discovered and include residues 192-202 [149] and 264-327 [145, 148, 150].

Most NAb target linear and conformational (discontinuous) epitopes located within the glycoproteins E2 and more specifically regions located in and close to the HVR1 of E2 [66, 69, 151] including the binding site for CD81 [152]. Located at the amino terminus of E2, HRV1 is an interesting target for NAbs as it is likely involved in virus entry due to it interaction with SR-BI [153, 154]. The most effective NAbs recognise epitopes located on the C-terminus of E2 where the E2/SR-BI interaction occurs [43, 154, 155]. Moreover, antibodies that recognise HRV1 show poor cross-neutralisation across different isolates of HCV of the same genotype due to the HRV1 high mutation rate [135, 156, 157]. NAbs targeting the less variable region (residues 412-421) directly downstream of HRV1 have also been found [158-160]. Monoclonal antibodies AP33, 3/11, HC33.4, HCV1 and H77.39 all recognise residues in the region 413–420 (Fig.1.3). However, this region has been suggested to be less immunogenic *in vivo* because NAbs targeting this region have been detected in only less than 5% of spontaneous resolvers [161]. As previously mentioned, E2 interacts with cellular proteins needed for viral entry including CD81 [162], SR-BI [38], NPC1L1 [37], and OCLN [31, 39]. Some NAbs (particularly AR3A, AR3B and AR3C) target epitopes spanning multiple conformation-sensitive regions, while NAbs 2/69, HC84.1, HC84.27 and 1/39 target epitopes in the region 427–447 (Fig. 1.3). A number of NAbs, including H77.31, H77.36, 1:7 and A8, have been found to target the CD81-binding loop of E2 (E2 amino acids residues at positions 519 to 535 of the HCV H77 consensus sequence) [48, 76, 142, 152, 163, 164].

NAbs contribute to the spontaneous clearance of infection, however the majority of infected patients progress to chronicity. Several mechanisms have been suggested to contribute to evasion of sterilising antibody-mediated clearance. Some of these include sequence changes, decoy epitopes, epitope masking, lipid shielding and cell-to-cell HCV transmission.

The high HCV sequence variability permits the virus to rapidly escape from Ab-mediated pressures. An estimated 10^{12} new HCV virions are believed to be generated daily in the infected liver [165] and thousands of mutant virions bearing all possible single and double nucleotide substitution pre-exist before treatment [166]. This viral sequence evolution, especially in E2, has been associated with NAb escape in chronic infection [73, 135, 140, 167]. The host NAb response lags behind the rapidly mutating E2 sequences within the quasispecies [73, 135], while continuing to exert selection pressure on viral variants and thereby contributing to selection of escape variants throughout the course of infection [73, 140].

The HRV1 region of E2 is highly immunogenic but is not vital for viral entry/infection. HRV1 deletion mutant have been shown to more sensitive to NAb responses, suggesting that HRV1 acts as an immunological decoy concealing epitopes sensitive to neutralisation [143, 168-170]. Moreover, NAb selection has been shown to drive HRV1 sequence evolution in chronically infected patients, while HRV1 remains stable overtime in Ig-deficient patients [171-173].

E1 and E2 proteins are heavily glycosylated with E2 containing up to 11 N-linked glycosylation sites most of which are highly conserved across the different genotype (Fig. 1.3). The glycan of the E1E2 ectodomain are estimated to contributing to almost half the apparent molecular weight of these proteins and have been shown to be essential for the structure and function of E1E2 and play an important role in viral entry [174-177]. These glycans are reported to prevent NAbs from accessing key neutralising epitopes [178, 179] as the removal of this glycan shield increases the sensitivity of HCVpp to NAb [176].

HCV is associated with high density lipoproteins [180, 181] causing interference with NAb activity. This association reduces HCV's buoyant density resulting in an increase in infectivity [182, 183] and cloaks the virus thus protecting it from NAbs [184, 185]. Additionally, the high-density lipoproteins (HDL) are thought interfere with NAb activity by interacting with the cell surface protein SR-B1 leading to enhanced viral entry into hepatocytes [66, 186].

HCV can spread by cell-to-cell transmission [180, 184] allowing the virus to bypass extracellular fluid, preventing NAb from accessing the viral particles [187, 188]. Induction of interfering Abs which compete with NAb for the same epitopes or is another mechanism thought to contribute to virus NAb evasion [189, 190]Overall, the findings of recent studies

suggest that the development of a NAb response correlates strongly with viral clearance. NAb responses alone were previously believed to be insufficient to confer protection against HCV, however passive transfer of broadly neutralising monoclonal or polyclonal NAbs to experimental animals protects against viral challenge [152, 191, 192]. These findings suggest that NAbs potentially play a vital role in neutralizing and reducing viral loads in infected HCV-infected patients and hence the induction of NAbs is an attractive target for prophylactic vaccine development [192]. An effective HCV vaccine would need to target multiple antigens from various genotypes in order to generate multi-antigenic, broad and highly reactive immune response during the initial stages of the infection before the virus can employ its many immune evasion mechanisms.

1.4.2.2. Cell-mediated immunity

CD4+ and CD8+ T lymphocytes are the main components in the cell-mediated immune response and appear 5-10 weeks post infection. HCV-specific CTL can eliminate virus from infected hepatocytes by secreting antiviral cytokines which are capable of inhibiting replication of the virus without destroying virus-infected cells and can eliminate infected cells by injecting toxins such as perforin (PRF), granzymes and granulysin into the virus-infected cells leading to their destruction [193]. CD4+ T cells on the other hand assist other immune cells in immunologic processes, including maturation of B cells into plasma cells and memory B cells, and activation of CD8+ T cells (see Fig. 1.4) [194].

Currently there are no correlates of protective immunity, however evidence suggests that during the acute phase of the infection, spontaneous HCV viral clearance has been linked to strong broadly directed and sustained HCV-specific CD4+ T cell responses [195, 196]. A weak antiviral CD4+ T cell response, on the other hand, is observed in HCV-infected patients who subsequently develop chronic HCV infection [197, 198]. Together these observations suggest that HCV specific CD4+ T cells play an important role in HCV control. Nevertheless, IFN-γ production by HCV-specific CD8+ T cells [26] and their accumulation in the liver [199] have also been shown to coincide with a rapid reduction in viraemia and the emergence of a HCV-specific CD4+ T cells response and finally viral elimination. The pivotal roles of CD8+ T cells in HCV clearance is also supported by the observation that following antibody (Ab)-mediated depletion of CD8+ T cells in chimpanzees, HCV viraemia increased for a prolonged time period [200, 201]. These data correlate with the concept that HCV-specific CD8+ T cells are the main antiviral effector cells, while HCV-specific CD4+ T cells have important helper functions and help to prevent viral escape from the CD8+ T cell response [202-204].

During acute infection, multi-specific, virus-specific CD8+ T cells are typically detectable in the peripheral blood 6-8 weeks after infection [93, 199] and this has been shown to be due to delayed priming of HCV-specific CD8+ T cells rather than delayed homing of the HCV-specific CD8+ T cells to the liver [205]. The mechanisms behind the CD8+ T cell failure are not well understood; however growing evidence suggests that CD8+ T cell exhaustion (characterised by impairment in CD8+ T cell effector functions) and viral escape contribute to this failure [93, 206, 207]. T cell exhaustion is defined by impaired CD8+ T cell effector functions, including a reduced ability of CD8+ T cells to secrete antiviral cytokines and to proliferate in response to antigen stimulation [206] as well as the co-expression of several inhibitory receptors such as programmed cell death 1 (PD-1), CD244 (also known as 2B4) and CD160 [207]. It is important to note that unlike CD8+ T cells, failure of CD4+ T cell responses are rarely linked to viral escape and this must be largely explained through other mechanisms [208, 209].

The low proportion of HCV-infected patients who successfully clear the virus illustrates the effectiveness of HCV immune escape strategies and makes development of an effective HCV vaccine a significant challenge, as these strategies may have important implications in vaccine development.

1.5. Model Systems to study HCV

1.5.1. In vivo models

HCV is known to only infect human and chimpanzees and for more than a decade the chimpanzee was the only animal model to study the course of HCV infection [210]. In contrast to human acute infection that can rarely be studied, the chimpanzee provided a model in which the progression of HCV disease could be monitored from beginning to end. Consequently, the chimpanzee model has provided valuable information on different aspect of the cellular immune responses and their role in disease outcome [211]. However, chimpanzees are expensive and due to ethical considerations are extremely limited in availability for experimental use.

Chimeric (xenograft) mice harbouring human hepatocytes are the most common small animal models in HCV research. These include urokinase plasminogen activator (uPA) and severe combined immunodeficiency disorder [212] mice (uPA-SCID) [213] as well as the Fah-/-Rag2-/-IL2rg-/-(FRG) model [214]. These mouse models are immunodeficient and characterised by hepatocyte degeneration due to an engineered genetic alteration [213, 215]. Once infected with HCV, these animals can be used to evaluate neutralisation and prevention of infection by antibodies, as well as the effects of antiviral compounds [152, 213, 215]. These animals have also contributed to our understanding of aspects of HCV biology such as viral entry, the role of

anti-HCV antibodies and infectious particle composition [191, 216, 217]. The uPA mice have been shown to be effective in the pre-clinical evaluation of antiviral compounds, and can generate similar outcomes to those observed in human clinical trials [218]. Combination therapy of NS3-4A and NS5B inhibitors using uPA mice was recently reported to eradicate HCV from animals with stable high-level viraemia [219]. This is in agreement with phase 2 clinical trials in which dual therapy with the NS5A inhibitor, daclatasvir, and the NS3 protease inhibitor, asunaprevir, led to a sustained virological response (SVR) [220] [221]. SVR is defined as the loss of detectable HCV RNA during treatment and its continuing absence at least 6 months after withdrawal of therapy. These findings support the utility of uPA transgenic mice for studying several aspects of the HCV life cycle and therapy.

Another animal model used for HCV studies is based on the deficiency of fumaryl acetoacetate hydrolase (FAH) mice crossed to an immunodeficient RAG2−/− IL-2Rγ^{null} background (FRG mice) to allow engraftment of human hepatocytes [222]. FAH is an enzyme in the tyrosine catabolic pathway and mice lacking the enzyme (FAH−/− mice), die from liver failure due to the accumulation of metabolites that are hepatotoxic [223]. However, treatment with a drug, Nitisinone (also known as NTBC), can block a different enzyme in this tyrosine breakdown pathway upstream of FAH and when mice are maintained on NTBC they remain fertile and viable [222]. Similar to studies with antivirals in the uPA model, treatment with IFN-α and a cyclophilin inhibitor transiently lowered viraemia in this model [214, 224]. Unlike the uPA mouse model which requires larger breeder colonies due to the infertility of homozygous mice, the FRG animal model has the ability to breed mutant mice on the drug NTBC, induce liver injury at any age by withdrawing NTBC, and is amenable to surgery on healthy mice [210, 225]. However, one drawback is the requirement in FRG mice for large numbers of human hepatocytes to achieve the high chimerism required for HCV infection. Unfortunately, the use of these mice in vaccine development is limited by a high mortality rate as well as the absence of a functional adaptive immune system [152, 215].

Other humanised mouse models such as AFC8-huHSC/hep [226] and Rosa26-Fluc [227] have been developed. The AFC8-huHSC/hep model is of particularly interest, as it allowed for dual engraftment of fetal hepatoblasts and autologous human hematopoietic stem cells giving rise to a liver chimeric animal harbouring components of a matching human immune system [226]. Nevertheless, these mice can currently only be used to study specific steps in the HCV life cycle and support limited (if any) viral replication.

Yu *et al*. recently developed a challenge model to examine the efficacy of HCV-specific cell mediated immunity elicited by a vaccine, including T cell trafficking to liver [228]. HCV

proteins were expressed in mouse hepatocytes after hydrodynamic injection of a plasmid encoding the HCV NS3/4A protein and secreted alkaline phosphatase (SEAP), which was detected in the mouse serum. Expression of NS3/4A in hepatocytes correlated with SEAP expression in serum. In a challenge experiment using this model, vaccinated mice showed accelerated clearance of SEAP and thus of NS3/4A positive hepatocytes and this coincided with an increased number of CD8+ lymphocytes in the liver [228].

Transgenic mice in a ICR background harbouring both human CD81 and occludin genes (C/O^{Tg}) were reported to be permissive to HCV infection at a chronicity rate comparable to humans [229, 230]. In this mouse model, HCV accomplished its replication cycle, leading to sustained viraemia and infectivity for more than 12 months post infection with expected fibrotic and cirrhotic progression. This recapitulates, for the first time in immune-competent mice, chronic HCV infection with a complete replication cycle and hepatopathologic manifestations [229, 230].

More recently, a HCV/GB virus B (GBV-B) chimeric virus carrying the major nonstructural proteins NS2 to NS4A (HCV NS2-4A chimera) was generated to infect common marmosets *(Callithrix jacchus)* [231]. Six of the seven HCV NS2 - 4A chimera-infected marmosets exhibited consistent viraemia while one showed transient viraemia during the course of follow-up detection. All infected animals with persistent circulating viraemia presented characteristics typical of viral hepatitis, including viral RNA and proteins in hepatocytes and histopathological changes in liver tissue. Viraemia was consistently detected for 5 to 54 weeks of follow-up. FK506 (Tacrolimus) immunosuppression facilitated the establishment of persistent chimera infection in the marmosets. An animal with chimera infection spontaneously cleared the virus in blood 7 weeks following the first inoculation, but viral-RNA persistence, low-level viral protein, and mild necroinflammation remained in liver tissue [231]. These chimera-infected marmosets represent as a suitable small-primate animal model for studying novel antiviral drugs and T-cell-based vaccines against HCV.

Animal models have contributed to studies of infection and are important for testing the *in vivo* relevance of *in vitro* findings. Chimpanzees, although no longer commonly used for HCV research, have particularly contributed to the study of the immune response, while humanised mice have been important for the study of the virus life cycle. Recently developed animal models, including the mice expressing humanised CD81 and Occludin genes and the HCV/GBV-B chimera-infected marmoset model represent potential tools for future development of novel antiviral drugs, immunotherapies, and vaccines against HCV infection.

1.5.2 In vitro models

1.5.2.1. Replicons

The development of subgenomic and selectable HCV replicons was as major breakthrough toward the establishment of a robust and reliable cell culture system for HCV [232]. The first HCV replicon, a nucleic acid that is capable of autonomous replication (designated Con1) was developed as a bicistronic construct with the first cistron encoding the neomycin phophotransferase (neo^r) gene under the control of the HCV IRES to allow selection for resistance against the drug G418. The second cistron encoded the HCV replicase genes (NS3-NS5B) directed by a heterologous EMCV IRES. Transfection of Huh-7 cells with *in vitro* synthesized replicon RNA and selection with G418, resulted in a few surviving cell colonies. This was thought to be partly because it was necessary for replicon RNAs to acquire adaptive mutations for efficient replication in the Huh-7 cells. Adaptive mutations tended to cluster within NS3, NS4B, NS5A and NS5B proteins [233]. Replication was enhanced with the introduction of adaptive mutations in the parental replicon, although some proved detrimental for infection *in vivo* [233-237].

1.5.2.2. HCV pseudoparticles (HCVpp)

HCVpp are engineered viral particles in which HCV E1-E2 glycoproteins are assembled on lentiviral or retroviral core particles in producer cells. Pseudotyping which is defined as the incorporation of heterologous viral glycoproteins into retrovirus particles, allowed the engineering and production of numerous chimeric VLPs derived from lentivirus (SIV) or oncoretrovirus (MLV). The development of infectious HCVpp by pseudotyping HCV glycoproteins onto a MLV-Gag retroviral core particle has enabled the study of viral entry [41]. A reporter gene, such as luciferase or green fluorescent protein (GFP) is also included to enable the detection and quantification of infected target cells. HCVpp harvested from transfected human embryonic kidney 293T (HEK293T) cells can be used to infect Huh-7 cells, while the infection process can be blocked by anti-E1 or anti-E2 specific antibodies [43, 155]. HCVpp representing glycoproteins of all major genotypes have been generated and have resulted in a further understanding of virus binding, attachment and internalization as well as identification of novel HCV receptors [40, 238].

1.5.2.3. Cell culture derived HCV (HCVcc)

The identification of JFH1, a genotype 2a subgenomic replicon that does not require adaptive mutations for efficient RNA replication in culture represented the first full-cycle HCV culture system [239]. It was later shown that transfection of JFH1 full-length genomes into Huh-7 cells resulted in the production of viral particles that were infectious in cell culture and animal

models [7, 44, 240]. However, infectivity titers attained with JFH1 were moderate. Much effort was made to improve the system and higher virus titers were produced when virus chimeras comprised of the JFH1 NS3 to NS5B regions fused to the core to NS2 region of other HCV isolates was found to be more infectious than authentic JFH1 [241]. Higher virus titers were obtained when JFH1 was propagated in Huh-7 cell clones such as Huh-7.5.1 and Huh-7-Lunet/CD81 high cell-lines which are more permissive for HCV replication [240, 242, 243]. The human hepatoma cell line LH86 was also shown to be susceptible to HCV infection [244]. Studies have shown that HCVpp and HCVcc share the same pH-dependent internalisation and fusion steps of the entry process [245]. Since the development of chimeric virus clones, viable JFH1 chimeras representing all major genotypes have been generated [246].

1.6. Diagnosis of HCV infection

HCV infection is rarely diagnosed during the acute phase of the infection. The diagnosis of HCV infection involves both serological and virological detection methods. Enzyme immunoassays are used to detect antibodies against different HCV epitopes provided that seroconversion has occurred and antibody titers are sufficiently high. However, the detection of HCV-specific antibodies cannot determine whether the infection is ongoing [247]. To confirm current infection, HCV RNA can be detected from the serum using RT-PCR, transcription mediated amplification (TMA) or branched deoxyribonucleic acid (DNA) (b-DNA) assay to provide both qualitative and quantitative analysis of viral load [247]. These methods can be used to determine the virus genotype, information which may be important to determine the dose and duration of treatment. Finally, a negative HCV RNA result is required on multiple occasions to confirm clearance of infection in a treated patient.

1.7. Treatment of HCV infection

Approximately 20% of acute HCV infections are cleared spontaneously by the host immune response, although this is extremely rare once the infection becomes chronic. Until recently the standard of care (SOC) for chronic HCV was a combination of pegylated-interferon-alpha and ribavirins (pegIFNα/RBV). The therapy is administered for a period of 24 (genotype 2 or 3) or 48 weeks (genotype 1, 4 or 6) [248]. This treatment resulted in a SVR in 40% to 50% (genotype 1 or 4) to 80% (genotype 2 or 3) of patients [249]. Recently, the IL28B gene has been reported to be involved in the immune response to certain viruses, including HCV. Three IL28B subtypes: CC, CT, and TT have been identified. Individuals with the CC genotype have a stronger immune response and respond better to treatment against HCV infection than those with non-CC genotypes [250, 251]. Other factors such as patient gender and age, HCV genotype, viral load and stage of liver fibrosis are also predictive of treatment outcome.

However increased side-effects including fever, headache, myalgia, haemolytic anaemia and severe depression have been reported with the combination of these drugs [252].

The mechanisms by which IFN and Ribavirin exert their therapeutic effect remains not well understood. IFN-α is believed to induce expression of numerous genes which trigger an antiviral innate response [253]. Ribavirin is a guanosine analogue and is only active against HCV when in combination with IFN-α. It has been hypothesised that Ribavirin may inhibit HCV replication after incorporated into nascent viral RNA by the HCV polymerase during RNA synthesis [253]. Ribavirin is also thought to act as RNA mutagen causing an accumulation of mutations that may lead to the collapse in viral fitness (termed 'error catastrophe' [254]).

New direct acting antivirals (DAA) are more effective, resulting in a cure for 90-95% of individuals with very few side-effects and allow shorter treatment periods (8-12 weeks). Telaprevir and boceprevir, which are inhibitors of the HCV NS3/4A protease, were the first DAAs to be licensed. They were administered together with the pegIFN/RBV combination as the SOC. This combination has been reported to improve the SVR but was still associated with side effect [255, 256] and therefore this combination is no longer recommended. Simeprevir and paritaprevir are new generation NS3/4A protease inhibitors and are administered in combination with other DAAs primarily to prevent the rise of drug resistance and increase antiviral potency. Another distinct class of DAAs that block HCV replication include NS5A inhibitors, namely, daclatasvir, ledipasvir, and ombitasvir. The NS5B polymerase inhibitors such as sofosbuvir and dasabuvir, represent the third class of DAAs that inhibit replication of viral RNA [257-259].

For HCV-infected patients who develop liver cancer, transplantation is the only treatment option available. Nevertheless, many infected individuals seek medical attention only when the disease has progressed to a late stage. Moreover, the high cost of therapy and the sophisticated clinical monitoring required for these therapeutic strategies makes their use, particularly in developing countries, unlikely. Therefore, the current standard of treatment and clinical practice is unlikely to have an immediate impact on the worldwide burden of HCV infection.

Vaccination has been the most successful and cost-effective strategy to prevent viral infection and virus-associated disease worldwide. The vaccine for another hepatotropic virus, hepatitis B virus (HBV), is estimated to have prevented over 1,000,000 deaths across the world as well as reduce the incidence of HBV-induced HCC [260].

1.8. Vaccines against HCV

Vaccination is a process of administering safe material (e.g. recombinant DNA, virus or proteins) to induce long-lived and protective adaptive immune responses against pathogens of interest. Many successful viral vaccines currently in use such as measles, mumps, rubella (MMR), oral poliovirus, varicella virus and yellow fever virus are predominantly based on live attenuated or inactivated viruses. Live attenuated viral vaccines have limited replication and pathogenic capacity in the host following immunisation but yet can induce protective immune responses against the respective wild-type virus [261-263]. These viruses are likely to be cytolytic and can possibly cause the release of specific PAMPs and/or damage-associated molecular patterns (DAMPs) from infected cells to trigger effective immune responses. However, live attenuated virus vaccines can cause severe complication for some patients (particularly immunocompromised patients) and secondary mutations have been reported to cause reversion of the attenuated virus to wild-type virulence [262, 264]. Compared to live attenuated virus vaccines, inactivated viral vaccines are rendered replication incompetent making them safer. However, inactivated viral vaccines lack the self-boosting quality of live attenuated virus vaccines as antibody titers decline over time and therefore require 3-5 doses [264, 265].

1.8.1. HCV vaccine clinical trials

Several prophylactic and therapeutic vaccines against HCV are currently under development. Research for prophylactic vaccines against HCV has particularly focused on the induction of NAbs in order to prevent in HCV-infected patients [192]. Other researchers are also developing therapeutic vaccines with an initial aim of priming and boosting functional T-cell responses in the periphery leading to the migration of these activated cells to the infected liver [266]. Currently several therapeutic vaccines are under development against HCV using a wide range of vaccine strategies. The composition of some of these vaccines are summarised in table 1.2.

Intercell AG (ICLL) (now Valneva Austria GmbH) in partnership with Novartis developed a therapeutic peptide vaccine (IC41) which consists of at least 4 HLA-A2-restricted CD8+ CTL epitopes and 3 highly promiscuous CD4+ helper T-cell epitopes derived from HCV genotype 1 core, NS3 and NS4 in combination with and T helper cell (Th)1/Tc1 adjuvant poly-L-arginine [268-270]. The vaccine is currently in its second phase II clinical trial. Although not significant, preliminary data in chronically infected genotype 1 patients who received 8 ID injections of the IC41 vaccine at biweekly intervals showed a 40% reduction of viral load compared with baseline [271]. The vaccine has also been shown to induce IFN-γ secreting CD4+ and CD8+ T-cells in patients with chronic HCV who had relapsed after- or failed to respond to- standard

IFN-based therapy [272]. The vaccine, however failed to delay relapse, which occurred in 8 patients (32%).

Table 1.1. Potential HCV vaccines in clinical phase development. Sponsor or company conducting the trial is listed along with clinical ID number (http://www.clinicaltrials.gov)

Type	Vaccine	Phase of clinical trial	Outcome	Sponsor/ company	Clinical ID/ reference	Initiation and completion date
Recombinant protein	HCV E1E2/MF59	Phase I	Induce antibody and E1/E2 specific T-cell response	NIAID	NCT00500747	August 2003 to August 2005
	Yeast expressing NS3-core protein (GI-5005)	Phase I	Elicit antigen specific T cell response	GlobeImmune	NCT00124215	June 2005 to February 2010
Peptide based vaccine	Core, NS3 and NS4 peptides with poly-L-arginine adjuvant (IC41)	Phase II	Strong T-cell response but transient effect on viral load	Intercell AG	NCT00602784	November 2002 to September 2004
	Peptide derived from Core protein (C35-44)	Phase I	Induced peptide specific CD8 T cell response in some patient		[267]	
DNA-based vaccine	ChonVac-C; NS3/4A DNA vaccine	Phase II	Transient effect on viral load	ChronTech Pharma AB	NCT01335711	April 2011 to June 2012
Recombinant viral vectored vaccine	MVA encoding NS3/4/5B (TG4040)	Phase II	Decrease in viral load in some patients	Transgene	NCT01055821	May 2010 to August 2013
	Adenovirus encoding NS3/5B (Ad6, AdCh3)	Phase I	Highly immunogenic	Okairos	NCT01070407	July 2007 to February 2011

GlobeImmune is developing GI-50005, a Tarmogen (targeted molecular immunogen) therapeutic vaccine consisting of whole, heat-killed recombinant *Saccharomyces cerevisiae* yeast expressing a HCV NS3-Core fusion protein [273]. Phase I treatment in chronic HCV-infected patients showed safety and significant immune responses correlating with viral load reductions. Another study was undertaken in 144 chronic HCV-infected patients to compare

efficiency of GI-5005 combined with PEG-IFN/ribavirin versus PEG-IFN/ribavirin alone. The combined therapy showed 15% greater efficacy in declining viral load and improved liver function for the treated patients (based on ALT normalizations) [274].

The vaccine TG4040 (Transgene) currently in clinical trials, uses a non-replicative modified poxvirus (Ankara strain) encoding HCV NS3, NS4 and NS5B from a genotype 1b viral strain [275]. The vaccine has been shown to have a good safety profile, induced HCV-specific cellular immune responses in 5 of the 15 patients (33%), and 8 patients showed a transient drop in HCV viraemia (from -0.52log to -1.24log) [275]. Recently, the efficacy, safety, and immunotherapeutic properties of TG4040 in combination with PEG-IFN/ribavirin was tested in patients with chronic HCV infection. The study showed that a higher proportion of patients with chronic HCV infection who received immunotherapy with TG4040 followed by TG4040 and PEG-IFN/ribavirin achieved a complete early virologic response (defined as HCV-RNA level less than 10 IU/mL after 12 weeks of PEG-IFN/ribavirin treatment) compared with patients who received only PEG-IFN/ribavirin therapy. This suggests that immunotherapies that activate T cells may be effective in patients with chronic HCV infection [276].

A DNA vaccine designed to induce T cell immunity has been developed by Tripep AB (Sweden). The vaccine known as ChronVac-C® is based on a codon optimised HCV NS3/4A gene (genotype 1) expressed under the control of the CMV immediate-early promoter. Results from a phase I/IIA clinical trial revealed that the vaccine was well tolerated and no adverse reactions were observed in any of the dosing arms. Two patients had viral load reductions which coincided with the development of HCV-specific T cell responses [277]. Recently the vaccine has been tested in mice to examine the concept of heterologous prime/boost vaccination by priming with ChronVac-C® and boosting with TG4040 [278]. Significantly higher levels of IFN-γ or IL-2 ELISpot responses were observed in the prime boost regimen compared with each vaccine alone, independent of the time of analysis and the time interval between vaccinations. A significant increase in polyfunctional IFN-γ/TNF-α/CD107a$^+$ CD8$^+$ T cells was detected following ChronVac-C/TG4040 vaccination (from 3% to 25%) [278]. These studies have provided proof-of-concept for DNA-based vaccines as an additional therapeutic agent to the current SOC, as well as combining two HCV therapeutic vaccines to improve their immunogenicity.

1.8.2. Preclinical vaccine research

Many successful viral vaccines currently in use (eg: oral polio, smallpox, and yellow fever vaccines) function by inducing strong NAb response against viral structural proteins to inhibit viral entry and thus prevent productive virus infection. Although many HCV vaccines strategies

focus on inducing T cell immunity, the ultimate aim for an effective preventative vaccine is to induce strong and broadly cross-reactive CD4+ and CD8+T-cell responses, and NAb responses [152, 279] capable of protecting against different genotypes. Viral proteins such as core, NS3 and NS5 [120, 280]) are commonly used in HCV vaccine design, as they are the prime targets of T cell-mediated immunity during natural infection. The two envelope proteins E1 and E2 targeted by NAbs, are also often included in order to induce both NAbs and T cell response [76, 281, 282].

1.8.2.1. Viral subunits

Various HCV subunit-based vaccines have been developed due to their potential to stimulate immune responses and control of HCV infection. The first encouraging results from subunit vaccine were obtained in the early 1990s after chimpanzees were immunised with purified HCV E1E2 glycoproteins [283]. Purified E1 and E2 mixed with MF59 adjuvant were also shown to produce NAb. All seven immunised animals exhibited strong humoral responses and five were completely protected from challenge after homologous HCV challenge. Although two of the chimpanzees became infected, the disease was less severe compared to control animals that were not vaccinated [283]. More recently the effect of immunisation with HCV E1E2 proteins (genotype 1a) with MF59C.1 adjuvant was investigated on 60 healthy volunteers [282]. The vaccine was safe and generally well tolerated, and detectable levels of NAbs as well as CD4+ T cell proliferation responses to E1E2 were reported.

A 40nm matrix composed of saponins, cholesterol and phospholipids (ISCOM) has also been used in HCV vaccine studies, using HCV core protein, purified from *E.coli*, absorbed onto ISCOMATRIX^{TM}. The resulting particles were able to stimulate strong long-lived, CD4+ and CD8+ T cell responses, and induced Th1 and Th2 cytokines as well as anti-core antibodies in rhesus macaques [280]. The efficacy and tolerance of a vaccine based on the HCV core protein with ISCOMATRIX^{TM} adjuvant was recently evaluated in thirty healthy volunteers [284]. Specific anti-core antibody responses were detected in 23 of the 24 vaccinated volunteers and T-cell cytokines in 7 of the 8 volunteers receiving the highest dose. A CD8+ T cell response was only detected in two of the volunteers [284].

Although these data are promising, lack of proper folding/functionality of the peptides as well as low immunogenicity are some of the major challenges facing the subunit approach [285]. In addition, difficulties involved in the purification steps as well as the extraction of sufficient quantities of these peptides would likely limit industrial scale production of vaccines using this strategy.

1.8.2.2. Viral vectors

Viral vector vaccines are recombinant viruses encoding targeted HCV proteins or peptides and have been shown to be highly immunogenic. These vectors have proven useful in stimulating immune responses against cancer specific antigens [286] as well as protection against animal viral diseases such as small pox and monkey pox virus [287, 288]. Pox viruses and adenoviruses are the major viral vectors used in HCV vaccine development. Since adenoviral infections are common, the vectors more commonly used are based on rare serotype such as the human adenovirus 6 (Ad6) or the chimpanzee adenovirus 3 (ChAd3) in order to avoid interference by pre-existing NAbs in vaccinated individuals [289].

The vaccines based on these vectors have been designed to elicit T cell immune responses and thus HCV NS proteins have been included in the vaccine candidates [289-291]. Immunisation with the Merck adenovirus serotype 6 (MRKAd6)-NSmut vector with or without a later boost with an electroporated plasmid DNA encoding the HCV NS proteins, resulted in the induction of broad CD4+ and CD8+ T cells as well as a 2 to 4 fold increase of the these responses following the plasmid DNA boost [292]. Although all animals became HCV-infected after viral challenge, those that received the vaccine presented a 100 times lower average peak of viraemia than controls [292]. A phase I clinical trial of a combination of both Ad6-NSmut and ChAd3-NSmut (HCV genotype 1b) was tested on 40 healthy volunteers. Immunised volunteers generated CD4+ and CD8+ T cells secreting multiple cytokines such as IL-2, IFN-γ, and TNF-α which targeted multiple epitopes and recognised heterologous 1a and 3a HCV strains albeit with differing magnitudes [291]. Together, these data indicate that the viral vector strategy can induce sustained T cell responses of a magnitude and quality associated with protective immunity and can be used for studies of prophylactic and therapeutic vaccines for HCV.

Although viral vectors have been shown to be effective and relatively safe, there are issues with large scale manufacturing and maintaining the genetic stability of the vaccine. The immune response against the viral vectors, although favourable in immune priming, can be a limitation due to pre-existing immunity in the host due to previous infection or vaccination. Viral vectors are therefore often not effective for repeated immunisation and more often viral vectors are used to boost an immune response after priming with DNA vaccine.

1.8.2.3. DNA vaccines

Recombinant DNA vaccines are bacterial plasmids constructed to express an encoded gene (i.e. antigen(s)) of interest) in cells. DNA vaccines are under development for several pathogens, cancer, autoimmune disorders and allergies. Currently, no DNA vaccine has been approved for use in humans, but four successful DNA plasmid products have been approved for animal use:

one for the treatment of West Nile virus in horses [293], one against hematopoietic necrosis virus in salmon [294], one for the treatment of melanoma in dogs [295], and a growth hormone-releasing hormone (GHRH) gene therapy for swine [296]. There are various reasons why DNA vaccines have not been approved for humans and one of the main reasons is lack of potent immunogenicity [266].

The use of DNA vaccines has several advantages compared to the traditional killed or live attenuated viral vaccines. DNA vaccines are known to induce cell mediated and humoral immune responses and result in antigen expression and processing in a way that mimics viral protein production after virus infection. Unlike protein or recombinant virus vaccines, DNA vaccines do not induce vector-specific immunity [297]. DNA vaccines produce immunological responses with none of the risks associated with live attenuated virus vaccines. Furthermore, DNA vaccines can be engineered to include specific immunogens that could optimise and amplify desirable immunological responses [298]. One concern has been the integration of the delivered DNA into the host genome, however studies have shown that the frequency of this event is much lower than that of spontaneous genome mutations, suggesting that this may not be a major concern [299-301]. DNA vaccines are highly stable (can be stored at room temperature), quite inexpensive and rapidly manufactured, therefore simplifying the handling and worldwide distribution of the vaccine and raising hopes that even developing countries can benefit from this type of vaccine [302, 303].

The concept for DNA vaccination against HCV is the production of non-viable, non-replicating, non-spreading antigens that can induce not only $CD4^+$ and $CD8^+$ T-cell immunity, but also B-cell immunity. After plasmid DNA is introduced into the body, it directly transfects muscle, skin or mucosal cells depending on the injection site, or circulating professional APCs. The endogenous expression and post-translational modification of vaccine antigen are major advantages of DNA vaccine priming since it mimics a natural infection, ie. endogenous expression of the immunogen. APCs have a dominant role in the induction of immunity of DNA vaccine by processing and presenting the antigen on MHC I molecules for activation of CD8+ T cells [1]. The majority of nucleated cells are able to present foreign antigens on MHC I and thus can be a target for CD8+ CTLs. Secreted antigens or apoptotic/necrotic bodies from transfected cells, are taken up or phagocytosed by APCs then processed and presented on MHC II for CD4+ T cells immune activation (Fig. 1.6).

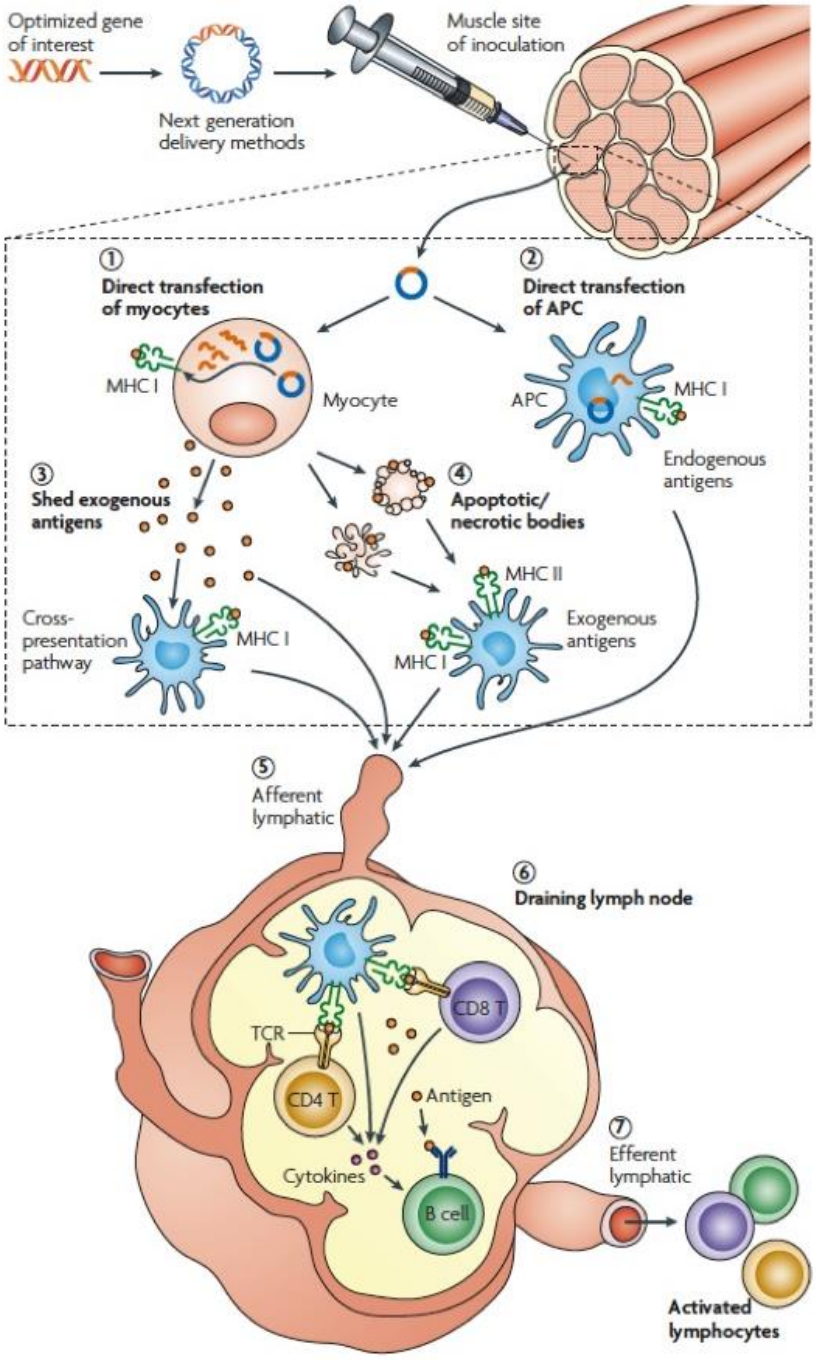

Figure 1.6. Induction of Cellular and Humoral Immunity by DNA Vaccination. Steps 1-4 represent the different ways that antigen becomes available to APCs. Steps 5-7 show migration of APCs to draining lymph node where they can present antigen and activate CD4 and CD8 T cells [1].

To ensure effective antigen expression within the host cells, a strong promoter such as the cytomegalovirus (CMV) promoter and an effective termination of transcription sequences such as the bovine growth hormone (BGH) polyadenylation signal are routinely used [304, 305]. Different organisms have different preferred codon usage and codon optimisation is another factor that has been shown to enhance both the gene expression levels and the immunogenicity

of DNA vaccines [306]. Codon optimisation has been reported to effectively enhance the overall protein production of several viral proteins including HCV NS3/4A and the human immunodeficiency virus (HIV) gag protein [307-309]. Vaccination with a synthetic codon-optimised NS5A (genotype 1b) construct resulted in high IgG titers as well as polyfunctional NS5A-specific CD8+ T cells responses in wild type and immunologically tolerant NS5A-Tg mouse models [310].

DNA vaccine strategies encoding HCV structural proteins have been shown to induce antibody as well as CD4+ and CD8+ T cell responses to HCV in mice [297, 302, 303, 311]. Despite having great potential in small animals, DNA vaccines have failed to demonstrate sufficient immunogenicity in human clinical trials and this is independent of body mass since DNA vaccines are now licensed for use in horses and salmon [312]. DNA vaccines however have been shown to be more immunogenic with alternative delivery methods [313], and as a prime-boost regimen coupled with a live virus vector [278, 292, 314-316]. DNA vaccination with HCV NS3 with adjuvants such as unmethylated CpG motifs and Quil A appear to induce strong antibody responses as well Th1 immune responses [317]. Phase I studies of a DNA vaccine containing a mixture of core/E1/E2-expressing plasmids with a recombinant HCV core protein, Co.120 (CICGB-230), showed safety, immunogenicity and improvement of liver histology in treated chronic HCV patients [318]. However, intramuscular (IM) immunisation with CICGB-230 of 15 chronically infected patients who were nonresponders to previous treatment with pegIFN-α/RBV resulted in no significant improvement in hematological and biochemical parameters including ALT levels between the baseline and post-treatment state, despite the induction of cellular immune responses [319]. Another HCV DNA vaccine encoding NS3/4A was efficiently expressed after codon optimisation and primed Th1 and CD8+ CTL responses in transgenic mice models [320, 321].

Taken together, DNA vaccination against HCV infection seems to be an efficient immunisation practice, particularly for therapeutic purposes. However, to date there is no DNA vaccine approved for use in humans and this is partly due to the low immune responses observed in larger animals including humans. Therefore, much effort has been made to find new techniques to deliver the DNA in more efficient way. Several other enhancements under investigation in addition to the delivery route include further optimisation of the vaccine construct as well as the use of adjuvants to enhance the immune priming. Advances in recombinant viral technologies have led to additional strategies that may generate safer approaches to viral based vaccine regimens.

1.9. Routes of immunisation of DNA vaccines

DNA-based vaccines are suitable for worldwide application because they are stable, heat-resistant, economical and easily manufactured. However as previously mentioned, the immunisation efficiency particularly in large animals is very low. Therefore, effective delivery is critical to the development of an effective DNA vaccine.

1.9.1. Intramuscular routes

Most vaccines, including the vaccines for influenza, hepatitis A, HBV, measles, mumps and rubella, are administered via the IM route [322, 323]. Traditionally the buttocks were thought to be an appropriate site for vaccination, but the layers of fat do not contain the cells necessary for the initiation of the immune response. Furthermore, it might take longer for the antigen to reach the circulation after being deposited in fat which might cause a delay in the antigen processing by APCs and eventually presentation to B and T cells [324]. Muscle cells express MHC I and MHC II molecules under inflammatory conditions *in vivo* and have been shown to possess the ability to present antigens to both CD4 and CD8 cells [325, 326]. However, the cellular biochemistry of antigen processing and presentation by muscle cells is still not clear [326]. Resident immune cells such as macrophages, DC and T cells are present in the muscle, but are relatively few in number compared to other organs which is one of the reasons that other administration routes are being investigated, particularly for DNA-based vaccines.

1.9.2. Subcutaneous routes

The epidermis and dermis are highly populated by DCs (Fig. 1.7) [8, 327]. The subcutaneous (SC) route of vaccination involves the delivery of vaccine into the region located beneath the dermis. The SC route has been used to administer various vaccines including the smallpox vaccine (vaccinia), measles vaccine and for immunotherapeutic vaccines to control asthma and allergy [8, 328]. The SC route has been shown to preferentially induce a Th2 type response when compared to the IM and intradermal (ID) routes [329]. A study in humans comparing administration of MVA vaccine via the SC and ID routes revealed that the dermal route induced equivalent NAb at a 10-fold lower dose compared with the SC route [330]. These data suggest that while the SC route is easier to access, it seems less effective that the ID route.

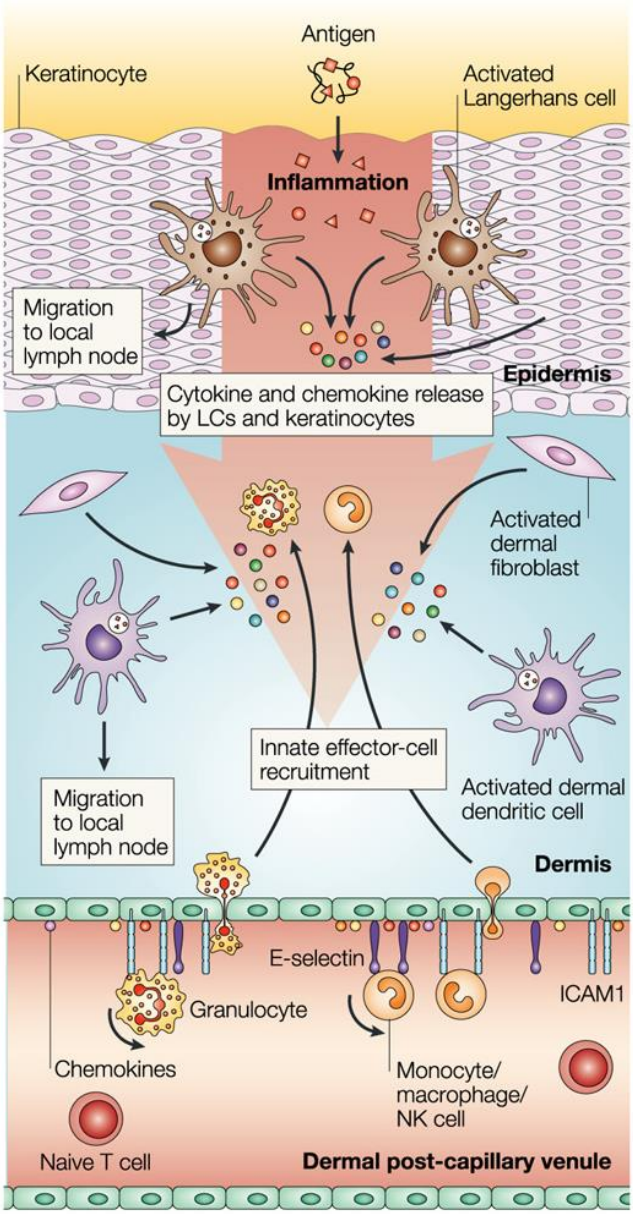

Figure 1.7. Innate immune response to antigen in the skin. Pathogen invasion leads to the release of primary cytokines and the activation of resident innate immune cells (Langerhans cells (LCs), dermal DCs and mast cells), stimulating downstream activation cascades. Activated LCs and dermal DCs mature and migrate to the draining lymph node, carrying antigen for presentation to T cells [8].

1.9.3. Intradermal routes

The rationale for ID vaccination is to deliver the antigen to the dermal layer of the skin located below the epidermis, that is rich in various immune cells including Langerhans cell, macrophages, mast cells as well as dermal DCs (Fig. 1.7). After administration via the ID route, immature DCs residing in the dermis can capture and process antigen, present peptides in the groove of MHC I/II on the cell surface before maturing and migrating to regional lymph nodes where they activate naïve CD4 and CD8 T cells [327]. Moreover, it has been shown that the recruitment of DC precursors from the bloodstream into the dermis and subsequent migration

to the lymph nodes is improved by administration of antigen via the ID route [331, 332]. Vaccination by the ID route has been shown to induce similar immune responses to IM vaccination with a lower dose of antigen [333]. Low dose ID influenza vaccines have been demonstrated to be safe, well tolerated and equally immunogenic as standard dose IM vaccines [334, 335]. ID injection of a DNA vaccine encoding the HCV NS3 fused to a DC-targeting molecule, scDEC, (a single-chain antibody against the murine DC cell surface molecule DEC205), in combination with electroporation resulted in robust NS3-specific antibody and T cell immune response [336]. Nevertheless administration of three doses of 4 µg of non-adjuvanted E1 vaccine via the ID route, induced much weaker humoral and cellular immune responses in healthy subjects and chronic HCV patients than the IM administration of 20 µg E1 formulated on alum [337].

Although ID vaccination is an appealing strategy to maximise the immune response to the antigen, it is technically challenging to deliver the vaccine formulation precisely. Currently researchers are investigating novel technologies to improve ID delivery.

1.9.4. DNA vaccine delivery systems

1.9.4.1. Physical DNA delivery

The principal of physical gene delivery systems is to use mechanical, ultrasonic, electrical, hydrodynamic or laser-based energy in order to create temporary weak points in the membrane of the targeted cell to make it more permeable for the DNA to enter by diffusion [338]. Electroporation induces uptake of injected DNA into the cell, by increasing the permeability of the cell membrane through exposure to controlled electric field [339, 340]. However, electroporation results in high cell mortality and therefore may not be suitable for clinical use. The Gene gun method uses accelerated particle carrier biocompatible heavy metals such as gold, tungsten or silver to deliver the transgene into the target cell and tissues [341, 342]. Gene-gun delivery improves DNA vaccine delivery compared to needle-based methods. However, particle bombardment is restricted to local expression in the dermis, muscle or mucosal tissue and surgery is often necessary for direct exposure of target tissue. As the skin is rich in resident DCs it provides increased transfection of the DNA directly into professional APC, regardless of whether the antigen is delivered via injection, gene-gun delivery, or electroporation.

1.9.4.2. Chemical DNA delivery

Liposomal delivery systems have emerged as one of the most versatile tools for the delivery of DNA vaccines. Liposomes are structures that consist of an aqueous compartment enclosed in a phospholipid bilayer. Depending on the composition and method of preparation, liposomes can take various forms, small unilamellar vesicles (with a diameter less than 50 nm) large

unilamellar vesicles (with a diameter of 50-500 nm) and multilamellar liposomes (with a large diameter up to 10,000 nm) [343]. Liposomes have been used for the delivery of anticancer drugs and can be used for DNA vaccine delivery by entrapping the vaccine inside the aqueous core or by complexing to the phospholipid lamellae [344]. Liposomes can load large amounts of DNA and increase the stability of DNA in the body, i.e. they can circulate in the bloodstream without causing any health concerns. However, there are many drawbacks that limit their application *in vivo* as they might form large aggregates at high concentration and also suffer from instability in the presence of serum. Cationic liposome formulations have been demonstrated by numerous studies to deliver different plasmid constructs to a wide range of cells, both *in vivo* and *in vitro.* They consist of three parts: hydrophobic anchor, a hydrophilic positively charged head, and a spacer (connecting the anchor to the head). Cationic lipids commonly used are 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), N-(1-(2,3-dioleyloxy) propyl)-N, N, N-trimethylammonium chloride (DOTMA), 2,3-dioleoyloxy-N-(2-(sperminecarboxamido)ethyl)-N, N-dimethyl-1-propanaminium (DOSPA), dioctadecyl amido glycil spermine (DOGS), and 3,(N-(N, N-dimethylethylenediamine)-carbamoyl)cholesterol (DC-chol) [345]. However, the use of these methods results in poor efficiency and cytotoxicity from the lipoplex-positive charge.

The most common method for the delivery of a DNA vaccine is injection with syringes and needles through IM, IV and ID routes. As route of immunisation affects the magnitude of the immune response, researchers have been developing methods to improve the delivery of vaccine antigen to target cells. Advances in the development of DNA-based therapeutics and chemical synthesis would allow the development of DNA delivery platforms that prevent degradation of DNA and facilitate targeting to specific tissues and cells.

1.9.4.3. Prime-boost regimens

Repeated vaccination is one approach used to generate strong immunity to specific pathogens [346]. Repeated administrations with the same vaccine (homologous boosting) have proven very effective. However, DNA vaccination using this approach has proven relatively inefficient in humans. Viral vector delivery has been more effective in priming a strong immune response, however this approach is relatively inefficient at boosting cellular immunity because prior or induced immunity to the vector itself might lead to a rapid clearance of the vector by the immune system. This may impair robust antigen presentation and the generation of appropriate inflammatory signals. One approach to circumvent this problem has been heterologous prime-boost strategies, using two different antigen-delivery systems generally administered weeks apart. The prime boost strategy has been shown to be effective in generating high levels of T-cell memory [346, 347]. Several studies have demonstrated the efficacy of prime-boost

vaccination with an initial vaccination using plasmid DNA to prime a broad immune response followed by a boost using a viral vector to generate cellular immunity to a variety of pathogens including HCV, HIV and other organisms [90, 348-350]. For vaccines designed to induce strong antibody responses, priming using plasmid DNA followed by a boost with recombinant protein might be effective. Ideally homologous immunisation with a competent adjuvant should be used to avoid delivery, manufacturing and distribution issues using several different vaccine components.

1.10. Adjuvants

Adjuvants have been used to improve the delivery and immunogenicity of vaccines. Adjuvants can stimulate both innate and adaptive immunity resulting in an earlier onset and prolonged duration of immunity, a higher magnitude of the immune response, and occasionally in a shift of the type of immune response. Adjuvants have been used to specifically target immune cells, recruit immune cells to the immunisation site or to facilitate the delivery of the vaccine antigen across barriers, such as the mucosal surfaces. Several adjuvants currently under development include: 1) immunostimulants that directly act on the immune system to increase responses to antigens including: TLR ligands, cytokines, saponins; 2) vehicles that present vaccine antigens (or immunostimulants) to the immune system in an optimal manner, including controlled release and depot delivery systems to increase the specific immune response to the antigen. Examples include: mineral salts, oil and water emulsions (such as MF59), liposomes, virosomes (nanoparticles made of viral proteins such as influenza hemagglutinin and phospholipids) biodegradable polymer microspheres as well as immune stimulating complexes (i.e. ISCOM, ICOSOMATRIX^{TM}). Some adjuvants currently used in approved human vaccines include Alum, MF59TM, MPL, VLP, Immunopotentiating Reconstituted Influenza Virosomes (IRIV) [351, 352].

1.10.1. Alum-based adjuvants

Aluminium salt based adjuvants, referred to as Alum, are components of several human vaccines including HBV [353]. It is believed that the aluminium salts may function by 1) causing a depot formation allowing the continuous antigen release; 2) particulate structure formation promoting antigen phagocytosis by APC such as DC, macrophages and B cells and 3) induction of inflammation resulting in recruitment and activation of macrophages and increased MHC class II expression and antigen presentation [354, 355]. Alum adjuvants have various advantages including safety, and often result in faster, higher antibody titers as well as long lasting antibody responses. However, their inability to induce Th1 or CTL responses that are required to control intracellular pathogens such as HCV makes alum less useful for DNA-

based vaccines. Other disadvantages of Alum adjuvants include loss of potency if frozen and causing granulomas at injection sites.

1.10.2. Other mineral salt adjuvants

Salts of calcium, iron and zirconium have also been used to enhance immune responses against antigens. Calcium phosphate in particular has been used in diphtheria tetanus pertussis (DTP) vaccines. Although calcium phosphate has similar properties to alum salts, it has the advantage of being a natural compound and is therefore exceptionally well-tolerated [356, 357]. It has the capacity to elicit enhanced systemic IgG responses and does not increase IgE production [357].

1.10.3. Complete Freund's adjuvant (CFA)

Complete Freund's adjuvant has been considered the most effective adjuvant available for raising antibodies in animal models for several decades. The adjuvant contains heat-killed mycobacteria, which is a primary agent responsible for stimulating antibody production. However, the use of CFA has been attributed to a number of undesirable side effects including increased pain, suffering and morbidity in inoculated animals and potentially serious health and safety threats [358]. Nevertheless, CFA is effective in stimulating cellular immune response and may lead to the production of IgG [359-361].

1.10.4. Adjuvant emulsions

Due to the shortcomings of alum-based adjuvants, other several novel adjuvants are under development to enhance the immune response to vaccines. These include oil-in-water (o/w) or water-in-oil (w/o) emulsions such as incomplete Freund's adjuvant (IFA) [362], montanide, MF59 and adjuvant 65.

The IFA water-in-oil emulsion is prepared from non-metabolised oils. The IFA induces immune responses through the formulation of a depot at the injection site and the stimulation of antibody production [363]. Adjuvant 65 is advantageous over the mineral oils used in IFA as it can be metabolised [361].

Montanide is a family of oil-based adjuvants that include ISA, 50V, 20G and 729. These have been used with natural, recombinant and synthetic antigens in mice, rats, dogs and cats. Montanide has been used in trial vaccines in humans against HIV, malaria and breast cancer [361].

Submicron oil and water emulsions such as MF59^{TM} contain squalene and varying amounts of muramyl tripeptide phosphatidyl-ethanolamine (MTP-PE) and is used as an adjuvant with influenza vaccines as well as HBV vaccines [364]. MF59 has been shown to induce higher antibody responses to HBV compared to alum in baboons and humans [365, 366]. The adjuvant

permits fewer doses, stimulates stronger antibody responses and generates marked memory responses, with a mixed Th1-Th2 cell phenotype [367-369]. MF59 induces local stimulation, recruitment of DCs, granulocytes, and differentiation of monocytes into DCs as well as increased uptake of antigen by DCs [370, 371].

Immunostimulatory complexes (ISCOMs) are nanoparticles composed of saponins (Quil-A) purified from the bark of a *Quillaja saponaria* tree, formulated with cholesterol, phospholipid, and antigen, held together by hydrophobic interactions. Protein antigens can be incorporated into the particles. An advantage of ISCOMs is their capacity to induce CD8+ specific cytotoxic responses. ISCOMs enhance antigen uptake and prolong retention by DCs in the lymph nodes, activate DCs and lead to increased antibody and T cell responses [372, 373]. The ISCOMATRIX adjuvant is identical to ISCOMs but does not contain antigen. This adjuvant can be mixed with antigens and like ISCOMs can target the antigen to APCs. ISCOMATRIX has been shown to induce good mucosal IgA responses. However, unlike ISCOM-based vaccines which induce a mixed Th1/Th2 response, vaccination with ISCOMATRIX induced a Th2-like response [374-376].

1.10.5. Cytolytic proteins as immune adjuvants

As previously mentioned DNA-based vaccines are poor inducers of T cell immunity. Deliberate induction of cell death to induce a cascade of different signals pathways that will activate innate responses in order to enhance antigen uptake by DCs can be used to improve immune responses and act as adjuvant for vaccines [115, 377, 378]. Following necrotic cell death, DAMPS such as heat shock proteins (HSP), Adenosine triphosphate (ATP), uric acid and high mobility group box 1 (HMGB-1) are released, providing danger signals to the immune system that can activate immature DCs [379, 380]. Dead cells are a rich source of DAMPs and the uptake of viral antigen-positive dead or dying cells by DCs and other APC represents a fundamental mechanism to elicit immunity against pathogens that do not intrinsically infect DC [381, 382]. Indeed, this mechanism has been investigated in cancer immunotherapy [383-387]. Currently there is little work exploiting this natural mechanism to ensure vaccine efficacy.

The body clears infected or damaged cells via apoptosis and necrosis. Although both apoptosis and necrosis ultimately lead to the same outcome (i.e., death of infected cells), the two processes differ significantly. In apoptosis, the entire affected cell, including the nucleus, separates into numerous fragment called apoptotic bodies. The genetic material of apoptotic cells simultaneously is fragmented and each apoptotic body is surrounded by a cell membrane. It is believed that the cells continue to produce proteins and adenosine triphosphate (ATP) and as a result, the apoptotic bodies contain intact, functional cell components [388]. Necrosis on the

other hand, is characterised by the loss of metabolic functions and the integrity of the cell membrane. During necrosis, the cell ceases to produce ATP, the organelles swell and become non-functional. Eventually, the cell membrane ruptures, resulting in the release of the cellular components (including DAMPs) into the surrounding tissues. This subsequently induces inflammation and immune activation [388, 389]. It is generally reported that in comparison to apoptotic cells which retain DAMPs within the cell, necrotic cells are more immunogenic as they release the cell contents including any immunogen, thereby ensuring DC targeting of the antigen. However, the mechanisms underlying the activation of DCs following both process are not well understood. Moreover, apoptotic cells that are not phagocytosed undergo secondary necrosis and currently there are no markers that can distinguish between primary and secondary necrosis following apoptosis [379, 389].

Both necrotic and apoptotic cells can be phagocytosed by DCs, however a secondary signal is required for DC maturation. It has been reported that exposure to necrotic but not apoptotic cells provides the necessary maturation signals [379]. Nevertheless, others have reported that increasing the ratio of apoptotic cells to DCs resulted in increased upregulation of markers of DC maturation including MHC class II and co-stimulation molecules CD80, CD86 and CD40, as well as secretion of inflammatory cytokines such as IL-12, TNF-α and IL-1β [390],suggesting that the number of dying cells is also important for complete activation of DCs. However, this can also be explained by the fact that high numbers of apoptotic cells might not be immediately phagocytosed by DCs and then undergo secondary necrosis, thus supplying the necessary signals for DC activation. Some cellular components released by necrosis that have been identified as a cause of inflammation include HMGB-1, uric acid and SAP130 [391-393]. A recently-characterized C-type lectin, Clec9A (also known as DNGR-1), has been identified as one receptor by which DC recognize a preformed signal that is exposed on necrotic cells [394]. Clec9A is found on mouse and human CD8α+ DC and was originally reported to be involved in endocytosis, activation, and pro-inflammatory cytokine production [394]. Clec9A is not required for uptake of necrotic fragments but is necessary for subsequent cross-presentation and priming of naive CD8+ T cells. Further investigation revealed F-actin as the protein that is recognised on necrotic cells and acts as a ligand that binds to Clec9A [395-397].

Collectively, these studies suggest a novel way to indirectly target DCs by inducing cell death of antigen-positive cells leading to the recruitment of the adaptive immune system during infection as a strategy to enhance immune responses generated by vaccines.

1.10.5.1. Cytolytic genes as cancer therapy

As noted above, necrotic cells are highly immunogenic and this mechanism has been investigated for cancer immunotherapy. The fundamental concept underlying cytolytic gene therapy is to target cancerous cells to directly eliminate the tumours and to induce an adaptive T cell response against tumour antigens that will lead to further elimination of cancer cells.

The most intensively studied gene system is the Herpes Simplex Virus Thymidine Kinase (HSV TK) and the prodrug Ganciclovir (GCV). In this system HSV TK is introduced to the target cells where it catalyses phosphorylation of GCV to ganciclovir monophosphate. Ganciclovir monophosphate is in turn converted to di- and tri -phosphate derivatives by cellular kinases. The triphosphate is incorporated into replicating DNA by cellular DNA polymerases, resulting in DNA chain termination and apoptosis [398, 399]. This system has been used to enhance tumour clearance by inducing cell death in tumour cell lines *in vitro*, and in animal models *in vivo*, as well as in various clinical trials for cancers such as glioblastoma and prostate cancer [387, 400-404]. Other suicide gene systems used for cancer therapy include: Cytosine Deaminase/5-FU [405], Carboxyl Esterase/Irinotecan [406, 407], Nitroreductase Nfsb/ 5-(Aziridin-1-Yl)-2 4-Dinitrobenzamide [408].

1.10.5.2. Cytolytic genes as adjuvant for vaccines

Although inducing cell death has been investigated in cancer immunotherapy, there are however only a few studies using this mechanism to enhance the immune response to vaccines. [383-387].

A DNA construct encoding Listeriolysin O fused to SIV gag was used to immunise mice resulting in enhanced immunogenicity to the vaccine [409]. Analysis of the immune responses to the DNA constructs showed higher levels of both CD4 and CD8 T cell responses against Gag. DNA constructs encoding secreted Gag proteins were also more effective in eliciting antibody responses against SIV Gag. These results demonstrated that the C-terminal segment of Listeriolysin O can be effectively employed to enhance both cellular and humoral immune responses in the context of a DNA vaccine. The authors hypothesised that Listeriolysin O may act on the endosomal membrane of APCs and allow the release of gag protein into the cytoplasm where it could be processed for antigen presentation [409]. They however did not investigate the exact mechanism of the toxin.

Other studies have focused on the use of molecules such as Fas to induce apoptosis at the site of vaccination [410]. By engineering Fas-mediated apoptotic death of antigen positive cells *in vivo*, the authors observed that the death of antigen-bearing cells resulted in increased antigen

acquisition by APCs including CD11c DCs. This strategy led to enhanced antigen-specific CTLs, and the amplification of Th1 type cytokines and chemokines.

Other approaches have focused on the expression of caspases 2 and 3 [381]. Administration of vectors co-expressing influenza virus hemagglutinin (HA) or nucleoprotein [411] genes and mutant caspase genes markedly increased both CD8+ and CD4+ T-cell responses, as well as B-cell responses to a lesser degree. The adjuvant activity was restricted to partially inactivated caspases that allowed immunogen expression before the generation of apoptotic bodies [381]. However, some studies have shown that preventing cellular apoptosis post vaccination with DNA enhanced the adaptive immune responses [412, 413] suggesting that apoptosis inhibits the immune response to vaccine antigens. Studies conducted in the Gowans laboratory have shown that targeted killing of antigen-positive cells via the necrotic or lytic pathway compared to the apoptotic pathway, resulted in improved immunogenicity of the DNA vaccines [414-418]. This suggests that the use of cytolytic genes represents an effective mechanism to induce inflammation and activate DCs which are crucial for the activation of the adaptive immune response to vaccine antigens [89, 416, 419].

1.11. Virus-like particles as a HCV vaccine candidate

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Preclinical Development and Production of Virus-Like Particles As Vaccine Candidates for Hepatitis C

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Hepatitis C Virus (HCV) infects 2% of the world's population and is the leading cause of liver disease and liver transplantation. It poses a serious and growing worldwide public health problem that will only be partially addressed with the introduction of new antiviral therapies. However, these treatments will not prevent re-infection particularly in high risk populations. The introduction of a HCV vaccine has been predicted, using simulation models in a high risk population, to have a significant effect on reducing the incidence of HCV. A vaccine with 50 to 80% efficacy targeted to high-risk intravenous drug users could dramatically reduce HCV incidence in this population. Virus like particles (VLPs) are composed of viral structural proteins which self-assemble into non-infectious particles that lack genetic material and resemble native viruses. Thus, VLPs represent a safe and highly immunogenic vaccine delivery platform able to induce potent adaptive immune responses. Currently, many VLP-based vaccines have entered clinical trials, while licensed VLP vaccines for hepatitis B virus (HBV) and human papilloma virus (HPV) have been in use for many years. The HCV core, E1 and E2 proteins can self-assemble into immunogenic VLPs while inclusion of HCV antigens into heterogenous (chimeric) VLPs is also a promising approach. These VLPs are produced using different expression systems such as bacterial, yeast, mammalian, plant, or insect cells. Here, this paper will review HCV VLP-based vaccines and their immunogenicity in animal models as well as the different expression systems used in their production.

Keywords: viral hepatitis, hepatitis C virus, preventative vaccination, virus-like particles, immune response, liver disease

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INTRODUCTION

Hepatitis C Virus is an enveloped positive sense single-stranded RNA virus that infects more than 170 million people (~2% of the world's population) (Bartenschlager et al., 2011). A majority of the individuals infected with HCV develop chronic hepatitis and a proportion will develop cirrhosis and liver carcinoma. HCV remains the most common reason for liver transplantation worldwide (Bartenschlager et al., 2011).

The introduction of highly effective direct acting antivirals (DAA) designed to inhibit the function of specific non-structural (NS) viral proteins critical to viral replication has resulted in high cure rates in most patients, a shortened duration of treatment and is associated with relatively

few side effects. However, the use of DAA is limited by the high cost (up to US$147,000 for 12-week treatment course) (Ahlén et al., 2013; Rosenthal and Graham, 2016). As a consequence, it is estimated that approximately ∼1% of those diagnosed with hepatitis C virus infection are treated annually in Australia (Commonwealth of Australia, 2014), while the global pool of HCV infected persons is increased by 3–4 million new infections each year (Gower et al., 2014). Furthermore, the World Health Organization (WHO) estimates that as of 2015 only 20% of those infected with HCV were aware of their diagnosis and only 7.4% of those diagnosed with HCV were placed on treatment worldwide (World Health Organization, 2017). Consequently, developing a safe, effective and inexpensive HCV vaccine is therefore necessary to control global infection and reduce the financial burden on healthcare systems.

However, the development of an effective HCV vaccine is hindered by several factors including the high genetic variability of the virus genome that gives rise to escape mutants which can evade both cellular and humoral host immune responses. Moreover, currently there are no suitable small animal models available that can mimic HCV infection of humans. Furthermore, humans and chimpanzees who have been previously treated and cured of hepatitis C could be re-infected after re-exposure to the virus (Farci et al., 1992; Bukh et al., 2008). Conversely, there is some optimism that the development of a successful HCV vaccine might be possible because a proportion of infected individuals (15–25%) clear the virus naturally and develop some immunity that is able to reduce the duration of viremia and the viral load in subsequent infections (Prince, 1994; Halliday et al., 2011). An understanding of the antiviral immune responses in these individuals and in the chimpanzee model have elucidated key mechanisms thought to contribute to the control of HCV infection. It was demonstrated that the induction and maintenance of strong CD4++ and CD8+ T cell immune responses against various viral epitopes are associated with the resolution of HCV infection (Forns et al., 2000; Thimme et al., 2001, 2002; Youn et al., 2005). It is now thought that a cell-mediated response alone is not sufficient and that the timely induction of cross-neutralizing antibodies (NAbs) is important for protection and in viral clearance (Farci et al., 1996; Yu et al., 2004; Osburn et al., 2014).

Licensed viral vaccines such as poliovirus, induce NAbs against viral surface proteins to provide protection. However, despite the development of an efficient system for HCV culture (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005), it is still not feasible to generate a vaccine based on killed or live-attenuated HCV due to the potential dangers associated with the use of such particles (Forns et al., 2002). These shortcomings have led to the development of experimental vaccines which include DNA vaccines, recombinant (non-pathogenic) vectors, proteins and virus like particles (VLPs). Despite the many obstacles that impede the development of these vaccines, several studies involving VLP-based vaccine candidates have already generated promising results in preclinical studies (Murata et al., 2003; Jourdan et al., 2006; Elmowalid et al., 2007; Patient et al., 2009; Roy and Noad, 2009; Garrone et al., 2011; Vicente et al., 2011; Bellier and Klatzmann, 2013; Huret et al., 2013; Beaumont and Roingeard, 2015). This article will review HCV vaccine strategies based on homologous or heterologous HCV VLPs (**Table 1**), plasmid DNA and viral vectors encoding HCV-VLPs (**Table 2**) as well as various expression systems (**Table 3**) utilized in the production of HCV VLP vaccines.

HCV VLP VACCINES IN PRE-CLINICAL STUDIES

HCV-Derived VLPs

Virus like particles are attractive candidates to elicit NAb responses as they structurally resemble and are much safer than the wild-type virus from which they are derived (Jourdan et al., 2006; Torresi et al., 2007; Roy and Noad, 2009). In addition to the hepatitis B virus (HBV) and HPV VLP vaccines, the VLP approach has been used to recently develop a chikungunya virus vaccine that was effective in non-human primates (Akahata et al., 2010). Self-assembled core-E1-E2 HCV-like particles (HCV-LPs) have been reported to generate HCV-VLPs with biophysical, ultrastructural and antigenic properties similar to those of the putative virion (Baumert et al., 1998, 1999). These HCV-LPs were generated in Sf9 cells from recombinant baculovirus encoding the HCV core, E1 and E2 proteins. Immunization with these recombinant HCV-LPs revealed that they can induce strong and broad polyclonal cellular and humoral immunity in mice (Baumert et al., 1999; Lechmann et al., 2001; Murata et al., 2003) as well as in chimpanzees (**Table 1**) (Elmowalid et al., 2007). The immunogenicity of HCV-LPs is strongly dependent on particle formation because vaccination with heat-denatured particles resulted in reduced antigen-specific T-cell and antibody responses (Lechmann et al., 2001). It is proposed that HCV-LP have the ability to interact specifically with dendritic cells (DCs) resulting in their activation and efficient antigen presentation, in contrast to denatured HCV-LPs (Murata et al., 2003; Barth et al., 2005).

Hepatitis C Virus-LPs that were produced in mammalian cells using an adenovirus-based system generated particles which were reported to resemble the native virions morphologically (Chua et al., 2012; Kumar et al., 2016). Vaccination with these adenovirus-derived HCV-LPs in combination with an anionic self-adjuvanting lipopeptide containing the Toll-like receptor (TLR) 2 agonist Pam_2Cys (E_8Pam_2Cys) resulted in significant HCV-LP and E2-specific antibody responses mice (Chua et al., 2012). Up to three doses of non-adjuvanted or traditional alum-adjuvanted HCV-LPs were essential to reach similar HCV-LP- and E2-specific antibody titers induced with a single dose of HCV-LP+E_8Pam_2Cys. In addition, these antibodies neutralized the binding and uptake of VLPs by human hepatocellular carcinoma (Huh-7) cells and of cell culture derived HCV (HCVcc). Significant numbers of antigen-specific antibody secreting cells were also detected in the spleen of HCV-LP+E_8Pam_2Cys vaccinated mice, correlating with the previous results (Chua et al., 2012). Moreover, vaccination of human leukocyte antigen (HLA)-A2 transgenic mice with this vaccine generated higher HCV-LP-specific IFN-γ-mediated responses compared to non-adjuvanted HCV-LPs (Chua et al., 2012).

TABLE 1 | Homologous and recombinant Hepatitis C Virus (HCV) virus-like particle vaccines.

Viral structural components	Expression system	Cells	Route of administration	HCV antigen	Animal studies	Reference
HCV-core	Insect cells	Sf-9	Intraperitoneal and intramuscular	Core, E1, and E2	Mice and chimpanzees	Baumert et al., 1999; Lechmann et al., 2001; Murata et al., 2003; Elmowalid et al., 2007
	Mammalian	Huh-7	Subcutaneous	Core, E1, and E2	Mice	Kumar et al., 2016
	Yeast	*P. pastoris*	Subcutaneous	Core, E1 and E2	Rabbits	
HBcore	Bacteria	*E. coli* (GI698 cells)	Subcutaneous	Core (98 aa) and NS3 (155 aa)	Mice	Mihailova et al., 2006
		E. coli (K802 cells)	Subcutaneous	Core (60 aa) also HBV-S1 (27 aa)	Mice	Sominskaya et al., 2010
HBsAg	Mammalian	Huh-7, HEK293T	Subcutaneous	E2 HVR-1 (36 aa)	Mice	Netter et al., 2001, 2003; Vietheer et al., 2007
		CHO cells	Subcutaneous	E1, E2, and E1E2	Rabbits	Beaumont et al., 2013, 2016; Beaumont and Roingeard, 2015
PapMV CP	Bacteria	*E. coli*	Subcutaneous	E2	Mice	Denis et al., 2007
MLV Gag	Mammalian	HEK293T	Subcutaneous, intramuscular and intradermal	E1 and E1E2	Mice and macaques	Desjardins et al., 2009; Garrone et al., 2011; Huret et al., 2013
			Intradermal	NS3	Mice	Huret et al., 2013
TMV CP	Plant cells	*N. benthamiana*	Intranasal	E2 HVR-1 mimotope R9	Mice	Nemchinov et al., 2000

TABLE 2 | Plasmid DNA and viral vectors expressing HCV virus-like particle vaccines.

Vector	Viral capsid components	Route of administration	HCV antigen	Animal studies	Reference
rVSV	HCV-core	Intravenous and intraperitoneal	Core, E1, and E2	Mice	Majid et al., 2006
Plasmid DNA	HCV-core	Intradermal and intramuscular	Core, E1, and E2	Mice	Murata et al., 2003
	MLV Gag	Intradermal	E1 and E2	Mice	Desjardins et al., 2009; Huret et al., 2013
			NS3	Mice	Huret et al., 2013

TABLE 3 | Comparison of VLP expression system.

VLP expression system	Merits	Demerits
Bacteria	Less expensive; simplicity of expression; fast growth rate; high-level expression; genetic stability; simple process scale-up	Lack mammalian-like PTM; Poor ability on immunogenicity; Presence of host cell-derived contaminants
Yeast	Less expensive; high-density fermentation; modification of the expression protein; moderately rapid expression; support most protein folding and PTM	High mannose modification; some secretory proteins cannot get ideal results; enhanced safety precautions are required
Insect cells	Moderately rapid expression; support most protein folding and eukaryotic-type PTM of the expression protein; works well for non-enveloped and enveloped VLPs, free of mammalian pathogens	High cost; difficult to scale-up; incomplete modification of proteins; low-level expression, contamination of product with enveloped baculovirus particles; perform simpler *N*-glycosylation compared to mammalian cells
Mammalian cells	Perform appropriate complex mammalian-type PTMs; perform authentic assembly and folding of recombinant proteins; works well for non-enveloped and enveloped VLPs	High cost; difficult to scale-up; lengthy expression time; low yield; vulnerable to infection with mammalian pathogens
Plant cells	Rapid expression; highly scalable; less expensive; free of mammalian pathogens; support most protein folding and eukaryotic-type PTM	Low yield; technical and regulatory issues

In a heterologous prime-boost strategy, immunization with recombinant adenoviruses encoding the HCV structural proteins as a final booster, following priming with HCV-LPs, resulted in enhancement of both antibody and T-cell responses (Kumar et al., 2016). Additionally, the sera from immunized mice reduced the binding of VLPs and the JFH1 strain of HCV to Huh-7 cells demonstrating the presence of NAbs (Kumar et al., 2016). Even though different studies have provided evidence that HCV-derived VLPs are a promising vaccine strategy, capable of eliciting HCV-specific humoral and cellular immunity, additional studies

are required to determine how best to induce highly protective, long-lasting immune responses.

Recombinant HBV-Based VLPs Containing HCV Epitopes

Hepatitis B virus-derived VLPs have been evaluated as carriers able to present heterologous epitopes (Chackerian, 2007), including HCV-derived antigens. In one approach, chimeric VLPs were generated by fusing target antigens to viral capsid proteins (CPs) capable of self-assembling into VLPs (**Table 1**). The p24/p27 protein of HBV surface antigen (HBsAg-S) has been reported to self-assemble into potent, non-infectious, secreted subviral particles and has been used in commercial vaccines against hepatitis B for over three decades. HBsAg-S particles have been designed as carriers of foreign epitopes inserted at the N-or C-terminus or into the external hydrophilic loop of the HBsAg-S (Patient et al., 2009). HCV-specific epitopes derived from the E2 protein hypervariable region 1 (HVR1) were inserted into recombinant HBsAg VLPs which were secreted from transfected mammalian cells (Netter et al., 2001; Vietheer et al., 2007). These particles were recognized by human anti-HCV-positive serum containing anti-HVR1-1b antibodies, suggesting the antigenic structure of the HVR1 region expressed on the particles' surface closely resembled the authentic structure (Netter et al., 2001). Subsequently specific antibody responses to HVR1 were efficiently induced (Netter et al., 2001; Vietheer et al., 2007). Furthermore, vaccination with a cocktail of HBsAg-S VLPs containing epitopes from either HCV-1a or -1b strains induced antibodies against both HVR1 epitopes that resulted in higher titers than those generated by immunization with the individual VLPs, suggesting a synergistic effect (Netter et al., 2001).

The insertion of the full-length HCV E1 and E2 envelope proteins into HBsAg VLPs was also shown to result in chimeric HBV-HCV particles similar in size and shape to the wild-type HBsAg subviral particles (Patient et al., 2009). Immunization of rabbits with these HBV-HCV VLPs resulted in high titers of cross-NAbs with the ability to neutralize various HCV genotypes (Beaumont et al., 2013). Recently, the immunogenicity of the chimeric HBsAg VLPs expressing E1 and E2 proteins as separate immunogens were compared with HBsAg VLPs bearing an E1E2 heterodimer (Beaumont et al., 2016). The E1- and E2- specific humoral responses induced in animals vaccinated with HBsAg VLPs expressing E1E2 in their heterodimer conformation, were significantly reduced compared to the responses in rabbits immunized with HBsAg VLPs expressing E1 and E2 separately (Beaumont et al., 2016). Additionally, the E1- and E2-specific antibodies showed increased cross-neutralization of various genotypes of heterologous HCV strains. These results confirmed previous observations (Garrone et al., 2011), that the E1 and E2 heterodimer is less immunogenic, possibly due to the conformational folding of E1 and E2 at the surface of the HBsAg particles contributing to the masking of certain immunodominant epitopes. Furthermore, although the folding of E1–E2 complex on the surface of the HBsAg particles was demonstrated, the structure of the E1E2 dimers on these particles might not be representative of dimers on the surface of native HCV virions. Nonetheless, these particles also generated significant levels of HBs-specific antibodies, further supporting the development of a bivalent HBV-HCV prophylactic vaccine capable of preventing primary infection with either of these two viruses (Beaumont et al., 2016). Administration of chimeric HBsAg-S/HCV VLP demonstrated that mice (Netter et al., 2003) and rabbits (Beaumont and Roingeard, 2015) with pre-existing anti-HBsAg antibodies successfully generated anti-HCV antibodies, therefore these chimeric particles can be used in populations with pre-existing immunity to HBV as a strategy to induce protective immunity to HCV. The encouraging results obtained so far support the development of HBV-derived VLP vaccines. However, additional studies are needed to assess the immunogenicity of these chimeric HBV/HCV particles in a larger animal model and also to develop means to further increase the broadly neutralizing characteristics of the resultant NAbs.

The HBV core protein (HBc) naturally self assembles into dimers and subsequently into VLPs and has been used as a VLP carrier for over three decades (Clarke et al., 1987; Borisova et al., 1993). Insertion of foreign sequences into the HBc gene does not impede particle formation making HBc suitable for the development of a VLP vaccine displaying a foreign antigen (Ulrich et al., 1998; Sominskaya et al., 2010). Segments of HCV genes encoding the core (98 aa) or NS3 (155 aa) proteins, containing B- and T-cell epitopes, were fused to the 3′ end of the HBc gene to generate chimeric HBc/HCV VLPs (Mihailova et al., 2006). These two proteins were chosen as immunogens because the induction of broad CD4+ and CD8+ T-cell responses to various HCV proteins, including core and NS3 is typically associated with resolution and control of HCV infection (Neumann-Haefelin et al., 2005). Administration of the HBc/HCV core particles induced moderately low antibody levels and proliferative responses of T-cell to HCV core epitopes, while vaccination with the HBc/HCV NS3 particles induced significant levels of anti-NS3 antibodies with no proliferative responses to HCV epitopes (Mihailova et al., 2006). However, antibodies to core and NS3 are believed to have no prophylactic value and are generally not considered to be protective in natural HCV infection. Furthermore, variations in the immune responses to the chimeric particles might be caused by alternative presentation of inserted HCV sequences in the context of the chimeric particles (Mihailova et al., 2006). Similarly, chimeric VLPs carrying a virus-neutralizing HBV pre-S1 epitope and a highly conserved N-terminal HCV core epitope at the C terminus of the truncated HBc protein (HBc-pre-S1-HCVcore) have been successfully generated (Sominskaya et al., 2010). Vaccination with these particles induced high T cell immunity similar to that induced by the monovalent HBc-HCV core particles, but induced low antibody responses to HCV core epitopes. It was argued that the core epitopes located at the C terminus were not exposed on the surface of the VLP and therefore were not able to induce strong antibody responses (Sominskaya et al., 2010). This further highlights the importance of the position of inserted epitopes within HBc in terms of the particle's humoral immunogenicity. Insertion of larger amino acid sequences would be beneficial for the development and production of a multivalent HCV vaccine carrying a larger number of epitopes coupled to one

carrier molecule and facilitate proper folding of conformational epitopes which is important for the generation of NAbs. However, inserting larger inserts within the HBc particles might affect the ability of the chimeric particles to drive insert-specific immune responses. Various studies have reported contradictory results regarding the maximum insertion capacity of HBc without affecting immunogenicity to the insert antigen and HBc's ability to successfully form particles. A maximum of 90 aa of human immunodeficiency virus (Kalams et al., 2013). Gag protein was reported to be tolerated while fusion of 317, 189, or 100 aa of the Gag protein downstream of aa 144 prevented self-assembly of the chimeric HBc particles (Ulrich et al., 1992). Yoshikawa et al. (1993), on the other hand, reported successful formation of HBc particles carrying a 720 aa segment composed of four copies of an 180 aa sequence derived from HCV core protein. However, the immunogenicity of these particles was not assessed in an *in vivo* model.

Recombinant Retrovirus-Based HCV VLPs

Complete glycoproteins can also be displayed in their native conformation on the surface of retroviral particles by a process known as pseudotyping. Infectious HCVpp which are engineered by pseudotyping HCV glycoproteins onto murine leukemia virus (MLV)-Gag retroviral core particles have been used to comprehensively study the early events of HCV infection, as well as the role of putative HCV receptors (Bartosch et al., 2003b). These particles are also commonly used to detect and measure anti-HCV NAb in HCV patients (Bartosch et al., 2003a; Drummer et al., 2003). HCV-pseudotyped retrovirus-derived VLPs devoid of a genome (HCV-retro VLPs) have been proposed as a vaccine platform. Studies of the molecular structure of these pseudoparticles described HCVpp and HCV-retro VLPs as regular 100 nm spherical structures comprised of the dense retroviral nucleocapsid surrounded by a lipid bilayer (Bonnafous et al., 2010; Garrone et al., 2011). Following priming with HCV-recombinant viral vectors, VLPs pseudotyped with E2 and/or E1 HCV glycoproteins generated significant anti-E2 and/or anti-E1 antibody titers in mice and macaques (Garrone et al., 2011). This highlighted the difficulty of inducing an anti-E1 antibody response, consistent with the findings that these antibodies are generally detected at low levels in patients (Leroux-Roels et al., 1996; Pestka et al., 2007). Of the regimens examined, only the VLPs induced significant levels of anti-E1 antibodies, which were generated provided that E1 was dissociated from E2. More importantly, boosting with the retrovirus-derived VLPs in a heterologous prime-boost combination with plasmo-retroVLP raised NAbs against HCV genotype 1a which cross-neutralized five other genotypes/strains (1b, 2a, 2b, 4, and 5) (Huret et al., 2013). No challenge experiments were conducted to assess the anti-HCV potency of these HCV-retro VLPs. Overall, retrovirus-derived VLPs pseudotyped with an assortment of virus envelopes, represent a versatile and efficient platform for vaccination not only against HCV but also against major infectious diseases such HIV/AIDS, yellow fever and dengue fever. However, despite these encouraging results, the use of animal retroviral particles has not been validated as a safe or effective preventative vaccine in humans, and scaling up the manufacturing process may present a limiting factor for the development of recombinant retrovirus-based HCV VLP vaccines.

Recombinant Vesicular Stomatitis Virus Vectors Expressing HCV VLPs

Recombinant vesicular stomatitis virus (rVSV) has been shown to be an effective expression vector in a number of different vaccine strategies (**Table 2**) (Schnell et al., 1996; Kretzschmar et al., 1997). There are several advantages of using rVSV encoding foreign viral proteins as a vaccine candidate. The virus has a low seroprevalence in the community and is not pathogenic in humans; the simple genome has only five genes (N, P, M, G, and L) and it does not undergo reassortment or integration (Johnson et al., 1966; Fields and Hawkins, 1967; Ezelle et al., 2002). Recombinant VSV has been evaluated as a candidate for HCV vaccination by encoding HCV core, E1 and E2 proteins (rVSV-C/E1/E2) which can self-assemble into HCV VLPs (VSV-HCV-VLPs) in baby hamster kidney fibroblasts (BHK-21 cells) (Ezelle et al., 2002). These VSV-HCV-VLPs showed similar ultrastructural properties to HCV virions (Ezelle et al., 2002). Vaccination with these particles induced core-, E1- and E2-specific cell mediated responses as well as anti-E2 antibody responses (Ezelle et al., 2002). However, it was argued that these particles may represent the endogenous viruses of BHK-21 cells known as intracisternal R-type particles, rather than the complete budded HCV-like particles (Blanchard et al., 2003). Subsequently it was demonstrated that the expression of HCV E1 and E2 by replication-competent and -defective (G-protein-deleted) VSV vectors resulted in correctly folded E1/E2 heterodimers (Majid et al., 2006). Nevertheless, this study only assessed the expression of the envelope proteins but did not provide a detailed characterization of the formed VSV-HCV-VLPs. Immunization with rVSVΔG-C/E1/E2 resulted in significant T cell responses to core, E1 and E2, along with anti-core and anti-E2 antibody responses (Ezelle et al., 2002; Majid et al., 2006). Neutralizing antibody responses were not assessed. However, immunization with rVSVΔG-C/E1/E2 protected mice against the formation of tumors expressing HCV E2 (CT26-hghE2t) and showed CT26-hghE2t-specific as well as E2-specific T-cell responses. Recombinant VSV expressing HCV structural proteins represent a versatile tool in vaccine development, which cannot only be used as a vector for the production of HCV VLPs in mammalian cells but also as a vaccine candidate itself. The recent effectiveness of an Ebola vaccine candidate rVSV-ZEBOV (Regules et al., 2017), comprising a live-attenuated VSV encoding the Ebola virus glycoprotein, in preventing Ebola virus disease in recently diagnosed patients, their contacts and contacts' contacts (ElSherif et al., 2017; Halperin et al., 2017; Henao-Restrepo et al., 2017) provides hope for the development of a VLP-based HCV vaccine using this strategy. However, further studies are required to better characterize and improve the production VSV-HCV-VLPs. The protective immunity generated by rVSVs encoding HCV core, E1 and E2 also needs to be evaluated

before these particles can be used as a vaccine against HCV infection.

DNA Vaccines Encoding Recombinant Retrovirus-Based HCV VLPs

DNA plasmids generating recombinant retro-VLPs (plasmo-retroVLPs) (Bellier et al., 2006, 2009; Desjardins et al., 2009) represent a HCV genetic vaccine circumventing the *in vitro* production and purification of retro-VLPs. These plasmo-retroVLPs have been engineered to produce *in situ* retroVLPs pseudotyped with E1 and E2 proteins after *in vivo* delivery of the DNA (**Table 2**). By doing so, this strategy combines the benefits of DNA vaccines such as the ease of development, low-cost large-scale production and stability with the immuno-stimulatory properties of VLPs, while circumventing the *in vitro* production of VLP vaccines. It has been shown that administration of plasmo-retroVLPs result in the induction of significantly higher antigen-specific responses and antiviral immune protection than plasmids that were mutated to prevent plasmo-retroVLP assembly (Bellier et al., 2006, 2009). It was also shown in heterologous prime-boost immunization strategies, that HCV-plasmo-retroVLPs were superior immunogens for boosting cellular and humoral immune responses in primed animals than plasmids unable to form E1/E2-pseudotyped retroVLPs (Bellier et al., 2009; Desjardins et al., 2009). A combination of two plasmo-retroVLPs vaccinations followed by a boost with *ex vivo* produced VLPs was shown to induce HCV-specific cell-mediated response and NAbs (Huret et al., 2013). Furthermore, vaccination with a cocktail of plasmo-retroVLPs pseudotyped with E1E2 from HCV genotypes 1a, 1b, 2b, 3a, 4c, and 5, and/or displaying NS3 antigen in CPs resulted in immune responses against the five HCV genotypes (Huret et al., 2013). A major advantage of plasmo-retroVLPs is that they can be re-administered for sequential vaccinations without the concern of pre-existing vector- associated immunity (Bellier et al., 2009). DNA-based vaccines, however, generally induce relatively weak immune responses to antigens and are therefore regarded as inferior compared to traditional vaccines such as subunit vaccines. As such, even the above strategy required boost immunizations with purified VLPs for maximum activity. The delivery route and/or method of DNA-based vaccines is also a critical factor in determining vaccination outcome. VSV-G specific plasmo-retroVLPs have been shown to induce higher levels of VSV-G-specific humoral and cell mediated immune responses following administration by needle-free intradermal (i.d.) injection compared to immunization with a gene gun (Bellier et al., 2009). Additionally, needle-free i.d. co-injection of cytokine genes or CpG sequences significantly increased VSV-G-specific NAbs levels (Bellier et al., 2009). Therefore, attempts should be directed toward the optimization of the delivery methods of DNA-based VLP vaccines and the addition or co-delivery of novel genetic adjuvants for maximum immune stimulation. Together, these results show that the plasmo-retroVLPs is a flexible platform to induce humoral and cellular immunity after homologous or heterologous prime-boost immunization and with further improvements, this strategy could be used as a promising approach to generate enhanced immune responses against HCV.

Recombinant Papaya Mosaic Virus-Based HCV VLPs

Virus like particles derived from plant viruses have also emerged as a promising strategy for the development of viral vaccines. The papaya mosaic virus (PapMV) CP has triggered much interest as an epitope presentation system. Several hundred units of the PapMV CP have been reported to assemble in an organized and repetitive manner into particles ranging from 60 to 100 nm in length following expression in *Escherichia coli* (Tremblay et al., 2006). A recombinant multimeric PapMV vaccine platform expressing a HCV-E2 peptide fused to the C-terminal PapMV CP was shown to trigger a strong humoral immune response in mice lasting more than 120 days against the CP and the E2 epitope (**Table 1**) (Denis et al., 2007). Notably, the same platform was less effective when inoculated as a monomeric protein, confirming the observation that immunogenicity is strongly dependent on the repetitive organization of the antigen (Denis et al., 2007). This VLP platform faces challenges which might limit its clinical development as vaccine, including the presence of bacterial RNA used a scaffold for PapMV CP self-assembly (Tremblay et al., 2006), as well as the possibility that antigen fusion might alter the antigen conformation or interfere with the protein assembly and VLP formation.

HCV VLP PRODUCTION SYSTEMS

Many expression systems for the production of VLPs are currently in use, including various species of yeast such as *Saccharomyces cerevisiae* (*S. cerevisiae*) and *Pichia pastoris* (*P. pastoris*), bacteria (mainly *E. coli*), the baculovirus expression vector/insect cell system (BEVS/IC), and various mammalian cell lines, as well as various plants (tobacco, lettuce leaves) (**Table 3**). The production system used depends on the need to retain more consistent original VLP formation and post translational modifications (PTMs, e.g., glycosylation and phosphorylation) while significantly reducing production times and costs. All production systems have advantages and drawbacks as discussed briefly.

Bacteria and Yeast

Bacterial systems are the most commonly used system to manufacture recombinant proteins and have been used to produce non-enveloped HCV VLPs. *E. coli*-derived HCV core proteins self-assembled *in vitro* into particles of approximately 60 nm in diameter (Kunkel et al., 2001). Lorenzo et al. (2001) on the other hand, produced VLPs in *E. coli* following expression of truncated HCV core protein consisting of the first 120 amino acids. Similarly, the recombinant HCV-E2 PapMV CP VLPs were also produced following expression in *E. coli* (Denis et al., 2007; Rioux et al., 2012). However, several factors including the lack of a mammalian-like PTM system means that this platform is not preferred for VLP production (**Table 3**). Additionally, the downstream processing of the VLPs is compromised by

the presence host cell derived toxins, heat shock proteins or chaperone proteins, increasing the final production cost (Zhang et al., 1998; Lai and Middelberg, 2002).

Yeast is another well-established platform used for recombinant protein expression and VLP production. Four licensed VLP-based vaccines, Engerix-B®, Hepavax-Gene®, Recombivax HB® and Gardasil® (Merck and Co), are manufactured using this system. The entire HCV core protein expressed in methylotrophic *P. pastoris* formed particles with similar structural properties as the native mature HCV nucleocapsid particles, and were successfully recognized by serum from a persistently infected HCV patient (Acosta-Rivero et al., 2001, 2004). More recently, VLPs of 70 nm in diameter were generated following expression of recombinant HCV coreE1E2 proteins in *P. pastoris* and were able to efficiently induce anti-CoreE1E2 antibodies in rabbits (Fazlalipour et al., 2015). However, concerns about appropriate protein processing, protein folding, and post translational modification, may direct the choice toward an alternative production system (Acosta-Rivero et al., 2004; Kushnir et al., 2012).

Mammalian Cells

Mammalian cell expression systems are favored because they are capable of performing appropriate complex mammalian-type PTMs and authentic assembly of recombinant proteins (Chua et al., 2012; Earnest-Silveira et al., 2016b; Kumar et al., 2016). Various HCV VLPs vaccine candidates have been successfully produced in mammalian cells including Chinese hamster ovary (CHO), Huh-7cells, and human embryonic kidney (HEK) 293T cells (**Table 1**). Immunization with recombinant HCV envelope glycoproteins produced in mammalian cells protected chimpanzees more effectively compared to those produced in yeast or insect cells after challenge with a homologous HCV isolate (Choo et al., 1994). Similarly, mammalian cell-derived recombinant envelope proteins have been reported to bind strongly to human cells compared to those produced in yeast or insect cells (Rosa et al., 1996). More recently VLPs produced in mammalian cells were found to be more immunogenic in a VLP prime/Rec-Ad-C-E1-E2 boost based heterologous strategy, when compared to VLPs produced using the baculovirus-insect cell system (Kumar et al., 2016). To avoid the deleterious effects of cesium chloride (CsCl), the use of iodixanol ultracentrifugation and stirred cell ultrafiltration resulted in the production of large quantities of HCV VLPs from various genotypes as a strategy for the manufacture of a quadrivalent mammalian cell derived HCV VLP vaccine (Earnest-Silveira et al., 2016a,b).

Plant Cells

Plant production platforms have many advantages compared to mammalian cells (**Table 3**). Plant-derived VLPs offer a new approach for oral delivery of vaccines. Plant expression systems are highly scalable, economical and free of mammalian pathogens. Additionally, plant production systems possess eukaryotic-like protein folding and PTMs similar to mammalian cells (Mett et al., 2008; Yusibov and Rabindran, 2008; Kushnir et al., 2012). Plant expression systems are suitable for the production of both non-enveloped and enveloped VLPs (D'Aoust et al., 2008; Santi et al., 2008). A tobacco mosaic virus (TMV)-derived vector encoding a HVR1peptide (containing a HCV neutralizing epitope) fused to the C-terminus of the B subunit of cholera toxin (CTB) was used to infect tobacco plants (*Nicotiana benthamiana*) (Nemchinov et al., 2000; Rybicki, 2014). Infected plants successfully generated TMV particles expressing HVR1/CTB (Nemchinov et al., 2000). The plant-derived HVR1/CTB reacted with anti-HRV1 monoclonal antibodies and sera from HCV-positive individuals infected with four of the major genotypes of HCV. Inoculating mice with plant extract resulted in the induction of both anti-CTB and anti-HVR1 antibodies capable of specifically binding to HCV VLPs (Nemchinov et al., 2000). A plant-derived HCV vaccine can reduce manufacturing costs associated with the production of conventional vaccines and the vaccine can be administered in edible plant part this would particularly helpful in reducing incidence of HCV in the developing world. However, to date no plant-derived VLPs have been licensed for human use. In addition, the dosage required for effective vaccination might vary depending on the plants used, ripeness of the fruit and the quantity of food consumed. Therefore, administration of edible vaccines requires to be standardized to evaluate the dosage requirement for effective vaccination. Also, certain plants and fruits are generally not eaten raw and cooking would result in denaturation of the protein and a reduction in immunogenicity to the vaccine. Most plants and fruits are susceptible to microbial infestation and this would affect the stability of the vaccine.

Insect Cells

The baculovirus-insect cell expression system has also been widely used for VLP production (van Oers et al., 2015). Insect cells have been employed to express several VLP-based vaccines, notably one of the current HPV vaccines, Cervarix®. The baculovirus–insect cell system can be used to manufacture non-enveloped and enveloped VLPs (**Table 3**) and various HCV VLPs have been successfully produced using this system (**Table 1**) (Baumert et al., 1999; Wellnitz et al., 2002; Choi et al., 2004; Zhao et al., 2004). The insect cell system can perform eukaryotic-type PTMs, support high expression levels of foreign proteins with the advantage of lacking mammalian pathogens (Roy and Noad, 2008; Kushnir et al., 2012). Expression of core, E1 and E2 proteins in the recombinant baculovirus system resulted in the formation of recombinant virus particles (Baumert et al., 1998, 1999, 2000) capable of inducing antibodies and cell-mediated immune responses after immunization in rabbits. A major disadvantage of this system is contamination of the product with co-produced enveloped baculovirus particles (Buonaguro et al., 2006; Palomares and Ramirez, 2009), which require the development of more complex and stringent VLP purification systems. Another limitation of this system is that insect cells perform simpler *N*-glycosylation compared to mammalian cells, which is troublesome in vaccine developments as correct glycosylation is often necessary for optimum immunogenicity of the vaccine antigen (Kost et al., 2005; Orlova et al., 2015).

DOWNSTREAM PROCESSING OF VLP VACCINES

The purity, potency and consistency of the particles and elimination of host cell and culture media contaminants are crucial for the downstream processing of VLP-based vaccines. In addition, the purification methods should be robust, cost-effective, scalable and preferably applicable to a wide variety of VLPs.

Virus like particles are purified using methods originally developed for the purification of viruses by which particles are purified based on their size and density using ultracentrifugation through sucrose or CsCl gradients (Vicente et al., 2011). However, ultracentrifugation-based methods are not practical for large scale vaccine development as they are non-scalable, tedious and highly labor intensive. Furthermore, use of CsCl is problematic due to its toxicity, reduced infectivity of viruses and particle deformation (Burova and Ioffe, 2005). Due to these limitations, there is currently a trend moving away from traditional VLP purification methods toward more scalable sophisticated techniques such as chromatography (Morenweiser, 2005; Vicente et al., 2011). Here, crude lysate is initially clarified by low-speed centrifugation or tangential flow filtration prior to chromatography (ion exchange, affinity, or size-exclusion). Chromatography provides a convenient and practical intermediate step for capturing and concentrating VLPs from cellular and media contaminants (Morenweiser, 2005).

CONCLUSION

The path to a HCV vaccine has been fraught with difficulties mainly because of the virulent nature of the virus and its ability to evade the immune responses due to its heterogeneity. However, advances in technology and our understanding of the natural course of HCV infection, the pathogenetic mechanisms, and the immunological markers which correlate with resolution of infection or protection, provide useful information for vaccine design. VLP vaccines have many favorable immunological characteristics making them promising HCV vaccine candidates. Currently, no VLP-based HCV vaccines have progressed to human clinical trials. However, VLP-based vaccines for the prevention of HBV and HPV infections have already been licensed, supporting the development of VLP-based vaccines for HCV. The HCV VLP-based vaccine candidates that have been developed so far have generated promising data in pre-clinical animal studies. It is widely known that several factors, including the expression system, will affect the quality and production yields of viral particles. Several expression systems have been effectively used to produce VLP vaccine candidate. Some VLP-based HCV vaccine candidates such as chimeric HBV/HCV VLPs can be produced using already established production systems such as bacterial and yeast. However, these particles can be contaminated with residual host cell factors such as lipids, nucleic acids and proteins that may stimulate innate immune responses and/or reduce the specific adaptive immune response and therefore may have a significant impact on VLP-based vaccine development that could present a bottleneck in their development. VLPs produced using mammalian cells offer an alternative system which supports appropriate complex mammalian-type PTMs and performs authentic assembly and folding of recombinant proteins and therefore generates VLPs that are very similar to the authentic HCV virion. However, difficulties in scaling up this system and high-production costs might direct the choice toward more cost effective alternative production system. Genetic vaccines that express HCV-recombinant VLPs, on the other hand are attractive candidates as the production of high quality plasmid DNA is simple and inexpensive to scale-up and could further advance the development of novel VLP-based strategies. Ultimately, it is hoped that newly developed VLPs can elicit specific and strong responses to HCV to prevent and contain infection. These encouraging results from pre-clinical animal studies suggest that it should be possible to develop such a vaccine although this may yet be some way off.

AUTHOR CONTRIBUTIONS

MM conceived the initial draft of the manuscript. DW, JT, EG, and BG-B revised many parts of the manuscript, and contributed to finalize the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

1.12. Thesis aims

The main focus of the work described in this thesis was to generate HCV DNA vaccines capable of inducing HCV-specific NAbs. These DNA vaccines encoded HCV structural proteins core/E1/E2 to induced HCV-specific immunity. To improve the immunogenicity of the DNA vaccine candidates, a cytolytic gene, PRF was encoded on the DNA constructs. Alternatively, in other studies HCV envelope proteins were fused to IMX313 or IMX313P to generate E1E2 heptamers. Detailed investigations of the subsequent immune responses to these optimised vaccines were then performed. This work attempts to realise the potential of DNA vaccines as a safe, stable and cost-effective prophylactic vaccine against HCV.

The specific aims of the thesis are to:

1) Construct a lytic DNA-based VLP vaccine encoding core/E1/E2 and PRF and evaluate the immunogenicity of these DNA vaccine in mice (See Chapter 3).

2) Construct DNA vaccines encoding E1E2 heptamers by fusing E1/E2 to IMX313 oligomerisation domains and assess their immunogenicity in mice (See Chapter 4).

3) Design DNA vaccines encoding E1E2 heptamers by fusing E1/E2 to the oligomerisation domain IMX313P and assess the effect of this oligomerisation on the subsequent immune response (See Chapter 5).

4) Evaluate the immunogenicity of DNA prime/DNA boost, DNA prime/E1E2 recombinant protein boost or DNA prime/HCV VLP boost regimens, using DNA encoding E1/E2 fused to IMX313P, E1E2 recombinant protein or HCV VLPs (see Chapter 6).

5) Assess the neutralising ability of the antibodies generated by the different prime/boost vaccination regimens (See Chapter 6).

Chapter 2. Material and Methods

2.1. Materials

2.1.1. Chemicals and Reagents

Chemical/Reagent	**Manufacturer**
2-mercaptoethanol	Sigma-Aldrich
30% Acrylamide/Bis Solution	BioRad
Absolute Ethanol	Sigma-Aldrich
Acetic acid	Thermofisher Scientific
Agar	AMRESCO
Agarose	AMRESCO
Albumin (bovine)	Sigma-Aldrich
Ammonium chloride	Sigma-Aldrich
Antarctic Phosphatase	NEB #M0289S
APS (Ammonium persulfate)	Sigma-Aldrich
Ampicillin	Sigma-Aldrich
Boric acid	Sigma-Aldrich
Brefeldin A	ebioscience
Bromophenol blue	Sigma-Aldrich
BSA (Bovine Serum Albumin)	Sigma-Aldrich
Calcium chloride	Sigma-Aldrich
Chloroform	Sigma-Aldrich
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride)	ThermoFisher Scientific
DMSO	Sigma-Aldrich
EDTA	Sigma-Aldrich
Ethanol	Sigma-Aldrich
Fetal calf serum (0.1um filtered)	ThermoScientific
GenElute	Life Technologies
Glucose	Sigma-Aldrich
Glycerol	Sigma-Aldrich
Glycine	Sigma-Aldrich
HEPES	Life Technologies
Hydrogen chloride	Sigma-Aldrich

Imidazole	Sigma-Aldrich
Isopropanol	Sigma-Aldrich
Kanamycin	Sigma-Aldrich
Lipofectamine® LTX with Plus™ Reagent	ThermoFisher Scientific
Magnesium chloride	VWR International
Magnesium sulphate	VWR International
Manganese chloride	Sigma-Aldrich
Non-fat dried milk	Black and Gold
optiMEM	Life Technologies
OptiPrep™ Density Gradient Medium (Iodixanol)	Sigma-Aldrich
Phosphate-buffered saline	Life Technologies
Potassium acetate	ACE chemical company
Potassium bicarbonate	Sigma-Aldrich
Potassium chloride	Thermofisher Scientific
Potassium hydroxide	Thermofisher Scientific
Protein G Agarose	Roche Diagnostic
Complete mini EDTA-free protease inhibitor	Roche Diagnostic
PVDF membrane	Millipore
RPMI 1640	Life Technologies
Sodium acetate	AMRESCO
Sodium azide	Sigma-Aldrich
Sodium Chloride (NaCl)	ChemSupply
SDS (Sodium Dodecyl Sulphate)	Sigma-Aldrich
Sodium hydroxide	Sigma-Aldrich
Sodium pyruvate	Life Technologies
N,N,N',N'-Tetramethylethylene-diamine (TEMED)	Sigma-Aldrich
Triton-X	BioRad
Trizma-base	Sigma-Aldrich
Trizol	Life Technologies
Trypsin	Life Technologies (Gibco)
Tryptone	Becton, Dickinson and Company
Western Lightning Ultra	PerkinElmer

Whatmann filter paper	Whatmann International Ltd
Yeast extract	Becton, Dickinson and Company

2.1.2. Equipment

Equipment	**Manufacturer**
Agarose gel imager	BioRad Gel DocXR system
Bacterial incubator	Labmaster Anax Division
Bacterial shaker	Ratek Orbital mixer incubator
Bacterial spectrophotometer	BioRad Smartspec 3000
Cell culture incubator	SANYO CO_2 incubator
EliSpot plate reader	Autoimmun DiagnostikaELISPOT reader
Flow cytometer	BD Biosciences FACScanto
Fluorescence microscope	Zeiss AxioLab inverted microscope
Heat block	BioRad Digital dry bath
Large centrifuge	Beckman T2-21M/E centrifuge
Large table-top centrifuge	Eppendorf centrifuge 5810R
Nanodrop spectrophotometer	Thermo Scientific
Platform shaker	Heidolph Instruments
Real-time PCR cycler	Corbett Rotor-Gene 3000
Small table-top centrifuge	Sigma 1-15PK
Western blot imager	FujiFilm LAS-4000

2.1.3. Kits

Kit	**Manufacturer**
E-Toxate (Limulus Amebocyte Lysate) test kits	Sigma-Aldrich
Pierce™ BCA Protein Assay Kit	ThermoFisher Scientific
PureLink® Quick Gel Extraction and PCR Purification Combo Kit	Life Technologies
PureLink® Quick Plasmid Miniprep Kit	Life Technologies
KAPA high fidelity PCR kit	KAPA Biosystems
KAPA Taq PCR kit	KAPA Biosystems
LDH Cytotoxicity Assay Kit	ThermoFisher Scientific
Pierce™ LDH Cytotoxicity Assay Kit	ThermoFisher Scientific

2.1.4. Cell lines

Cells	Description
Huh7	Human Hepatoma cell line
HEK-293T	Human Embryonic Kidney cell line

2.1.5. Antibodies

2.1.5.1 Primary Antibodies

Antibody	Class	Raised in	Source	Catalogue number
Anti-core	Monoclonal	Mouse	ViroStat	1851
Anti-E1	Monoclonal	Mouse	ViroStat	1879
Anti-E2	Monoclonal	Mouse	ViroStat	1876
Anti-HCV	Pooled HCV genotype 1 patient sera	Human	Inhouse	
Anti-PRF	Monoclonal	Rat	Abcam	CB5.4
Anti-actin Ab-5	Monoclonal	Mouse	BD Biosciences	612656

2.1.5.2 Secondary Antibodies

Antibody	Source	Catalogue Number
Alexa-fluor (AF) 555 conjugated donkey anti-goat IgG	Invitrogen	A21432
AF 555 conjugated goat anti-human IgG	Invitrogen	A21433
AF 488 conjugated goat anti-human IgG	Invitrogen	A11013
AF 555 conjugated goat anti-mouse IgG	Invitrogen	A21127
Cy5 conjugated goat anti-rat IgG	Life Technologies	A10525
Alexa-fluor 555 conjugated goat anti-human IgG	Invitrogen	A21431
Horse Radish Peroxidase (HRP) conjugated goat anti-human	Millipore	AP112P
HRP conjugated goat anti-mouse IgG	Antibodies Australia	GAM.2.2
HRP conjugated goat anti-rabbit IgG	Antibodies Australia	GAR.2.2
HRP conjugated goat anti-mouse IgG	Invitrogen	62-6520
HRP conjugated donkey anti-goat	Life research	L00180020
HRP conjugated goat anti-mouse IgG	Antibodies Australia	GAM.2.2
HRP conjugated rabbit anti-mouse IgG2a	ThermoFisher scientific	61-0220
HRP conjugated sheep anti-mouse	GE Healthcare Life Sciences	NA931-1ML

2.1.6. Solutions

2.1.6.1. Bacterial Expression

Solution	**Components**
Luria-Broth (LB)	10 g tryptone, 10 g NaCl, 5 g yeast extract, 950 ml H_2O
LB-agar	10 g tryptone, 10 g NaCl, 5 g yeast extract, 950 ml H_2O, 15 g agar
SOC Media	20g Tryptone, 5g Yeast Extract, 0.58g NaCl, 0.19g KCl, 2.03g $MgCl_2$, 2.46g $MgSO_4$, 3.6g Glucose (Final volume 1 L using MQ H_20)
TfbI solution	2.94g KAc 30mM, 7.45g KCl 100mM, 1.47g $CaCl_2$ 10mM, 9.85g $MnCl_2$ 50mM, 150ml Glycerol 15% - Adjust the pH to 5.8 with acetic acid (Final volume 1 L using MQ H_20)
TfbII solution	0.209g MOPS 10mM, 1.1g CaCl2 75mM, 0.075g KCl 10mM, 15ml Glycerol 15% - Adjust the pH to 6.5 with KOH (Final volume 100 ml using MQ H_20)
Psi-a Media	6g Yeast Extract, 24g Tryptone, 6g $MgSO_4$ - Adjust the pH to 7.6 with KOH (Final volume 1.2 L using MQ H_20)
Psi-b Agar	2.8g of agar added to 200ml of Psi-a media

2.1.6.2. DNA Manipulation

Solution	**Components**
DNA 6× loading dye	25 mg bromophenol blue, 25 mg xylene cyanol FF, 3.3 ml glycerol, 6.7 ml ddH2O
1× Tris/Borate/EDTA (TBE) buffer	10.8 g Tris, 5.5 g Boric acid, 4 ml 0.5 M Na2EDTA (pH 8.0), 900 ml ddH2O

2.1.6.3. SDS-PAGE

Solution	**Components**
Running Gel Buffer	40 mM Tris, 185 mM Glycine, 0.1% SDS
Resolving Gel Buffer	2 M Tris HCl pH8.8, 10% SDS, 30% Acrylamide
Stacking Gel Buffer	2 M Tris HCl pH6.8, 10% SDS, 30% Acrylamide
5× SDS loading buffer (Reducing)	100 mM Tris-HCl pH6.8, 2% SDS, 2mL 10% glycerol, 5% β-mercaptoethanol, 1µg/ml bromophenol blue

2.1.6.4. Western Blot Analysis

Solution	**Components**
Running buffer	6.25mL pH8 Tris, 6.25mL pH8.8 Tris, 10mL 10% SDS, 14.4g glycine, to 1L MQ H_2O
Wet Transfer Buffer	14.4g glycine, 3.03g Trizma Base, to 1L MQ H_2O
Skim milk solution	200mL PBST (1xPBS + 0.05% Tween), 10g skim milk powder, 1g BSA

2.1.6.5. Cell Lysis

Solution	**Components**
ACK Lysis Buffer	0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA (pH 7.2-7.4)
NP-40 Lysis buffer	150 mM NaCl, 1% NP-40, 50 mM Tris-Cl (pH 8.0)
RIPA Buffer, Triton X-100	50 mM Tris-HCl (pH 7.4), NaCl 150 mM, 1% Triton X-100, 0.5% Sodium deoxycholate, 0.1% SDS (pH Value: 7.4)

2.1.6.6. Tissue Culture

Solution	**Source**	**Catalogue number**
Trypsin-EDTA (0.25%), phenol red	Life Technology	25200-056
Dulbecco's modified Eagle's medium (DMEM)	Life Technology	21969-035
Minimum Essential Medium (MEM)	Life Technology	11095-080
penicillin/streptomycin	Life Technology	15140-122

2.1.7. Oligonucleotide Synthesis

Oligonucleotides were purchased from GeneWorks and Integrated DNA Technologies

2.2. Methods

2.2.2. Molecular Cloning

2.2.2.1 DNA Constructs

All DNA vaccine constructs were based on two vectors; the pVAX1 plasmid (Life Technologies) and a bicistronic vector, pJ, generated by modifying the pVAX1 vector to insert the SV40 promoter, a novel multiple cloning site and the SV40 polyadenylation site [415]. Codon-optimised genes (Gene Art, Germany) encoding Core, E1 and E2 (GenBank accession number AF139594.2) were used as template in a series PCRs.

2.2.2.2. Primer Design

Primers were designed from each of the genes of interest to amplify the entire gene and introduced start (ATG) or stop (TGA) codons where necessary. A Kozak sequence (GCCACCATGG) was also introduced by the primers for enhanced mammalian cell expression and novel restriction enzyme sites were also introduced so that the DNA amplicon of the gene of interest could be digested and ligated into the plasmid backbone. The primers also contained a short sequence of approximately 20 bases complementary to the target DNA. Primers were purchased from Geneworks Pty Ltd or Integrated DNA Technologies and were <100 bases. Primers were designed to avoid primer dimerization, primer hairpins, and multiple binding sites on the template, and aimed for a melting temperature (Tm) between 55 - 65 °C with the primer pair Tm to fall within 5 °C of each other. The Tm was calculated using the following equation: Tm = 2 °C (# A + T pairs) + 4 °C (# G + C pairs).

A complete list of primers used in this thesis is shown in appendix I.

2.2.2.3. PCR Amplification

2.2.2.3.1 PCR Amplification

For direct sequencing, the required gene or gene fragments were amplified using the KAPA high fidelity (HiFi) Taq polymerase enzyme PCR kit (KAPA Biosystems). Each reaction mix contained a final volume of 50 µl and consisted of 1X KAPA HiFi Buffer, 0.3 mM of each dNTP, 0.3 µM forward primer, 0.3 µM reverse primer, 10 ng DNA template and 0.5 U KAPA HiFi DNA polymerase. Each reaction was amplified at:

Number of Cycles	1	30			1
Temperature (°C)	95	98	Up to 65*	72	72
Time (minutes)	2	0.3	0.2	1 minute/kb	5

* The annealing temperature is dependent on the length and composition of the primer.

Following amplification, the entire reaction was subjected to agarose gel electrophoresis (section 2.2.2.5) and the band of interest purified by gel extraction (section 2.2.2.6) using the PureLink® Quick Gel Extraction and PCR Purification Combo Kit (Life Technologies).

2.2.2.3.2 Overlapping PCR Amplification

The overlapping PCR amplification was performed in three stages: extension PCR, overlap PCR, and finally purification PCR. During an extension PCR, individual fragments encoding genes with overlap sequences to allow hybridisation were amplified. PCR products were then electrophoresed on a 0.8% agarose gel, which was stained with gelred.

The PCR products purified by agarose gel electrophoresis were used as template DNA for the second stage-overlapping PCR without primers and subjected to the following amplification conditions: 1 cycle of 95°C for 2 minutes; 35 cycles of 95°C for 30 seconds, 72°C for 1 minute, and 72°C for 3 minutes; and a single extension of 72°C for 5 minutes. In this process, strands with overlapping complementary base pairs (20 bases) acted as primers for each other. In the third PCR, the combined (larger) fragment gene was amplified using primers on the extreme 5' and 3' ends of the PCR product. The forward primer contained a Kozak sequence, a start codon and restriction enzyme and a reverse primer contained a stop codon and restriction enzyme sites.

2.2.2.4. Restriction Enzyme Digestion

PCR products of the genes of interest and the plasmid backbone were both digested with the appropriate restriction enzymes (NEB). Restriction enzyme digests were performed for at least 3 hours at 37°C (to ensure complete digestion) unless otherwise specified by the manufacturer. Reactions were typically carried out in 50µL volume using 10 units of each enzyme per µg DNA. All reactions were performed using the appropriate enzyme buffers and BSA if necessary. The vector backbone was de-phosphorylated using Antarctic Phosphatase (NEB). Following digestion, the gene insert and the vector backbone were ligated (section 2.2.2.7).

2.2.2.5. Agarose Gel Electrophoresis

This method was used to isolate and purify DNA fragments amplified by PCR or DNA fragments produced by restriction enzyme digestion. Typically, larger fragments (>500bp) were separated on 0.8% agarose gel and smaller fragments (<500bp) on 2% agarose gel. The agarose gels were prepared in 1× TBE buffer. DNA fragments to be loaded on the gel were first mixed with 0.1 volume of 6× DNA loading dye and gels were typically electrophoresed at 100 V in 1× TBE buffer. DNA fragments were electrophoresed alongside 1kb ladder (GeneSearch Pty

Ltd). To excise digested DNA fragments and PCR products, the fragments were visualised using long wave UV light followed by purification (section 2.2.2.6.).

2.2.2.6. Purification of DNA from Agarose Gel

Following agarose gel electrophoresis, the band of interest was extracted and purified using the PureLink® Quick Gel Extraction and PCR Purification Combo Kit (Life Technologies). Excised gel slices containing digested DNA fragments were dissolved in buffer 1 (solubilisation buffer) at a ratio of 1:3 and incubated at 50°C for 15 minutes. The dissolved DNA fragment was then mixed with 1 gel volume of isopropanol to increase DNA yield. DNA in the gel solution was then bound to a quick spin column by centrifugation at 13,000 rpm for 1 minute. The column was washed once with 750 µl buffer PE (washing buffer) and centrifuged again at 13,000 rpm for 1 minute. DNA was eluted from the column by the addition of 30-50 µl of buffer EB (elution buffer) followed by further centrifugation at 13,000 rpm for 1 minute.

2.2.2.7. DNA fragment Ligation

Gel purified DNA fragments following restriction enzyme digestion were ligated for 16 hours at 16 °C in 10µl reactions containing: the gene insert and plasmid backbone mixed at a ratio of 3:1 (at a final concentration of 100ng) with 1× ligase buffer and 2 units of T4 DNA ligase. Final DNA concentrations were calculated using the following formula: ng insert = ((insert size (kb)/vector size (kb)) × ng vector) × 3. Following ligation, 2 µl DNA was used for transformation into competent *E. coli* (section 2.2.2.9).

2.2.2.8. Preparation of heat-competent bacterial cells

DH5α cells were streaked from a frozen stock on a psi-b plate and incubated overnight at 37°C. A single colony was picked and inoculated into 10 ml psi-a medium with shaking at 37°C until OD550 to 0.3 was reached (about 4 hours). 5ml of the culture was then added to 100ml of pre-warmed psi-a medium and incubated at 37°C with shaking until the OD550 was 0.48 (about 3 hours). The culture was chilled on ice for 5 minutes followed by centrifugation at 4500 rpm for 5 minutes at 4°C. The pellet was resuspended in 40ml chilled Tfb-I solution and chilled on ice for 5 minutes followed by centrifugation at 4500 rpm for 5 minutes at 4°C. The pellet was then resuspended in 4ml chilled Tfb-II solution and incubated on ice for 15 minutes before being snap frozen in liquid nitrogen in 50µl -100µl aliquots and stored at -80°C.

2.2.2.9. Bacteria cells Transformation

The ligated DNA was then transformed into heat-competent DH5α *E. coli* cells. 2 µl (50ng) of plasmid or ligated DNA was added to 50 µl heat-competent *E. coli* cells. The cells were transformed using the heat shock method by incubating the mixture of DNA and *E. coli* cells

on a preheated heat block at 42°C for 45 seconds. Transformed cells were then resuspended in 0.5 ml SOC media and incubated at 37°C with shaking at 180 rpm for 1 hour before being plated on kanamycin (50µg/mL) Luria-Broth (LB)-agar plates and grown overnight at 37°C. DH5α cells transformed with 3:1 ligation mix were compared to a 0:1 ligation mix to calculate colony background levels.

2.2.2.10. Positive Colony Selection

The identification of bacterial colonies containing the correct insert was performed by colony PCR, diagnostic digest or DNA sequencing.

2.2.2.10a. Colony PCR

Colonies were selected and diluted in 50µl of MQ H_20. Colony PCR was performed using KAPA taq polymerase (KAPA Biosystems). The total number of colonies tested depended on the number of colonies on the vector-only negative control plates. The colony PCR master mix contained 0.25 U KAPA taq polymerase, 1X KAPA taq buffer, 0.2 mM of each dNTP, 0.4 µM forward primer, 0.4 µM reverse primer and 2 µl diluted bacteria colony.

Thermo Cycler Conditions using the DNA Engine PCR machine (BioRad) were as follows;

Number of Cycles	1	30			1
Temperature (°C)	95	95	Up to 65*	72	72
Time (min)	2	0.3	0.30	1 min/kb	2

*The annealing temperature is dependent on the length and composition of the primer.

The colony PCR products were analysed by electrophoresis on a 0.8% agarose gel to confirm the correct product size.

2.2.2.10b. Restriction Enzyme Digestion of Cloning Products

Restriction enzyme digestion of the cloned products was performed to verify positive colonies. DNA isolated with a mini-preparation kit (see section 2.2.2.11) was digested with the appropriate restriction enzyme to identify the insert in the vector. The digested DNA was analysed by electrophoresis on a 0.8% agarose gel to confirm the size of the insert and vector.

2.2.2.11. Small Scale Plasmid Preparation (Mini-preparation)

A positive bacterial colony was picked from the antibiotic-supplemented agar plate and grown in antibiotic-supplemented LB media for 16 hours at 37 °C with vigorous shaking at 180 rpm. Plasmid DNA was purified from these bacterial cultures using the Invitrogen Quick plasmid mini-preparation kit protocol (Life Technologies) according to the manufacture's protocol. The DNA concentration was determined by a Nanodrop spectrophotometer. Purified plasmids were

re-digested with appropriate restriction enzymes and analysed by agarose gel electrophoresis to confirm that the gene of interest was present. The correct sequences of the positive clones were further confirmed by DNA sequencing (section 2.2.2.13).

2.2.2.12. Large Scale Plasmid Preparation from Transformed Bacteria

A single colony was picked from a freshly streaked selective agar plate to inoculate a 500 ml culture of LB with kanamycin (100µg/ml) and cultured overnight at 37°C with vigorous shaking (180 rpm). A large-scale DNA preparation was then made from the bacterial pellet harvested by centrifugation at 3000g for 10 minutes at 4°C. The pellet was resuspended in 30 ml buffer P1 (cell suspension buffer) by pipetting,30 ml of buffer 2 (lysis buffer) was added to the suspension and mixed by vigorously inverting the tube 5-6 times. The suspension was incubated at room temperature (RT) for 5 minutes before adding 30 ml of buffer 3 (precipitation buffer) and further mixing by inversion. The lysate was then centrifuged at 3000g for 10 minutes and the supernatant was transferred to new labelled tubes. To precipitate the DNA, 1 volume of isopropanol was added to each tube and mixed by inverting the tubes for a few times before being centrifuged at 3000g at 4 °C for 10 minutes. The supernatant was discarded and the pellet air dried for 10-20 minutes. To remove cellular proteins, the pellet was resuspended with 1-2ml TE buffer + RNase (100 µg/ml) and pipetted up and down several times to completely dissolve the pellet. An equal volume of phenol: chloroform (Life Technologies) was added and gently mixed to extract the DNA followed by centrifugation at 15,000g for 15minutes at 4°C. The top layer of the mixture (containing DNA) was transferred to a clean tube and the DNA was precipitated by adding 4mls of 95% ethanol, 100ul (1/10 volume of 3M sodium acetate), 10µl glycogen (20mg/ml) (Life Technologies) and incubated at -80°C for 30minutes followed by centrifugation at 15,000g for 30 minutes at 4°C. The DNA pellet was air-dried and dissolved in 1-2ml of TE buffer. To remove endotoxins, 100-200µl of 3 M sodium acetate solution was added to the extracted DNA and incubated on ice for 5 minutes. 200-400µl of cold Endotoxin Removal Solution (Sigma Aldrich cat. No E4274) was then added to the mix and incubated on ice for 10 minutes followed by an additional incubation at 37 °C for 20-30 minutes (or until the phases separated). The mixture was then centrifuged for 5 minutes at 3000g and the upper (colourless) phase carefully transferred to a clean centrifuge tube. The endotoxin removal steps were repeated twice. To precipitate the DNA, 0.6 volume of 2-propanol was added and mixed before centrifugation at 15,000 x g for 30 minutes at 4 °C. The DNA was then washed by adding ~500µl cold 70% ethanol and centrifuged at maximum speed for 10 minutes at 4°C and the pellet air-dried. In the final step the DNA pellet was dissolved in 1-2ml of endotoxin free water or TE buffer. The DNA concentration and endotoxin levels were

determined using a Nano-Drop and E-TOXATE (Endotoxin testing) kit (Sigma #ET0200) respectively.

Alternatively, DNA constructs were prepared using the QIAGEN Plasmid Giga Kit (Qiagen #12191) following the manufacturer's instructions.

2.2.2.13. Nucleotide Sequencing

Sequencing of the genes of interest in the DNA vaccine constructs was performed by SA Pathology IMVS DNA sequencing facility. 1µM of a single forward or reverse primer, and 200ng of plasmid DNA were required for the sequencing reaction. The BLAST nucleotide alignment was used to confirm that the sequence in the plasmid DNA construct matched the known sequence of the gene of interest.

2.2.1. Cell Culture and transfections

2.2.1.1. Cell culture and storage

All components of cell culture media were supplied by Invitrogen. Huh7 and HEK-293T cells were grown in $DMEM_{complete}$ (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) (heat inactivated at 56^{o}C for 30 minutes), 1% penicillin/streptomycin) and maintained at 37^{o}C in an atmosphere of 5% CO_2. Cell lines were typically grown in T75 or T150 tissue culture flasks. At reaching about 90% confluency, cells were washed gently with DPBS followed by their removal with trypsin (Life Technology). Cells were resuspended in 10mL of $DMEM_{complete}$ before re-seeding or use in experiments.

For cell storage, cells were trypsinised, pelleted and re-suspended in 1 ml of 10% DMSO in FCS and transferred to a cryovial. The cells were then transferred to a freezing container (Nalgene) and stored at -80 °C overnight then transferred to liquid nitrogen.

Vials of cells were thawed in a 37°C water bath and then transferred to a 10mL falcon tube; 9 ml of pre-warmed media was then added and the cells were pelleted by centrifugation at 1500 rpm for 5 minutes. The supernatant was removed, and the cells were re-suspended in fresh media and plated.

2.2.1.2. Transient cell transfections

HEK293T cells were seeded at 6×10^5 cells per well in a 6 well plate and allowed to grow and adhere overnight, then maintained at 37°C in an atmosphere of 5% CO_2 resulting in approximately 60-70% cell confluency on the day of transfection. Cells were then transfected with 2.5µg DNA/well using Lipofectamine LTX according to the manufacturer's protocol. A control well transfected with DNA encoding the reporter gene eGFP was used to confirm transfection efficiency. The transfected cells were incubated for 36 hours at 37 °C with 5% CO_2

and protein produced in transfected cells was detected by western blot, flow cytometry or immunofluorescence.

2.2.3 Protein Detection

2.2.3.1 Protein purification and quantification

2.2.3.1a Protein Purification by Affinity Chromatography

The HCV E1E2 proteins were purified under native conditions on a nickel-nitrilotriacetic acid (Ni-NTA)-agarose column according to the manufacturer's protocol (Qiagen). Briefly, HEK293T cells were transfected with the construct encoding 6 x His-tagged secreted E1E2 polyprotein (p-tPAtE1tE2-Histag, see chapter 3 – section 3.3.6.2 and chapter 4 – section 4.3.1) in serum free media. The cell culture media was harvested 48 hours post transfection, centrifuged for 5 minutes at 4000 rpm to remove detached cells before filtration through a 0.45µm filter to remove remaining cell debris. Proteins in the clarified supernatants were concentrated by ultrafiltration through a 70,000-molecular-weight cutoff filter (70K MWCO; Amicon) for 20 minutes at 4000 rpm. The concentrated proteins in the supernatant was applied on the Ni-NTA column (Qiagen). Following three washes by buffer B (30 mM Immidazol, and pH 8), purified protein was eluted with 300 mM imidazole elution buffer (pH 8). Purified E1E2 proteins were analysed by PAGE (section 2.2.3.2) and western blot (section 2.2.3.3).

2.2.3.1b Protein quantification

Protein concentration was determined by bocinchonimic acid (BCA; ThermoScientific) or Bradford (Bio-Rad protein assay) assays. The BCA protein assay was conducted according to the manufacturer's instruction using standards of bovine serum albumin (BSA) in a range of 25 – 2000µg/mL. The absorbance was measured at 562 nm on the FLUOstar OPTIMA plate reader (BMG LabTech). For the Bradford assay, 10µL of each sample was transferred into the wells of 96well plate. 10µL of the buffer was used as a blank. 200µL of Bradford dye reagent were added to each well, mixed with the proteins and incubated at RT for 5 minutes followed by OD measurement of at 595nm. Measurements were performed in duplicate or triplicate for each sample and the average was taken for the calculation of the protein concentration. Unknown sample concentrations were calculated based on the standard curve of the protein standards according to the manufacturer's instructions.

2.2.3.2 SDS-PAGE

For SDS-PAGE, cells were lysed directly in Triton-X 100 lysis buffer. The lysates were then spun briefly (13,000 rpm for 1 minute) to remove cell debris and the clarified lysates were mixed with 5×SDS loading buffer. For secreted proteins, cell culture media was harvested, centrifuged to remove detached cells before filtration through a 0.45µm filter to remove

remaining cell debris. The proteins in the clarified supernatants were then concentrated by ultrafiltration through a 70,000-molecular-weight cutoff filter (70K MWCO; Amicon). All samples were denatured by boiling at 95°C for 5 minutes before use. Resolving gels were prepared using acrylamide solution at a final concentration of 10-14% in 1 x resolving gel buffer. Stacking gels were prepared using acrylamide at a final concentration of 5% in a 1 x stacking gel buffer. Polymerisation of gels was initiated by the addition of APS (0.1%) and TEMED (to 0.8%). A 10-tooth comb was typically used to form wells in the stacking gel. Denatured proteins were loaded onto the gel alongside a protein marker (Sigma) for size determination. Fractionation was performed at 120 V in gel running buffer. Upon separation, gels were removed from the apparatus and the proteins were transferred onto polyvinylidene difluoride (PVDF) membrane.

For non-denaturing PAGE, the samples were lysed in native sample buffer (Bio-Rad) and were not denatured before electrophoresis. Native gel electrophoresis was performed in the same manner as SDS PAGE, except that SDS was omitted from the gels and buffers to ensure that proteins retained their activities.

2.2.3.3 Western Immunoblotting

Proteins separated on polyacrylamide gels were transferred to PVDF membrane using a BioRad transblot semi-dry blotting device. Transfer was carried out at 25 V for 15 minutes. Following transfer, membranes were incubated for 1 hour at RT or overnight a 4°C in PBST containing 5% dried milk powder to block non-specific binding of antibody. Membranes were washed 3 times in PBST and probed with the appropriate antibody (see section 2.1.5 for a list of the antibodies used) diluted in skim milk for 1 hour at RT. The membranes were washed as above and incubated with the appropriate HRP conjugated secondary antibody (diluted in skim milk) for 1 hour at RT. Finally, the membranes were washed as above and bound antibody was detected using Western Lightning Ultra (PerkinElmer) reagents I and II in equal ratio. Bands were visualised by LAS-4000 westernblot imager (FujiFilm).

2.2.3.4 Indirect Immunofluorescence

Immunofluorescence (IF) was used to visualise the intracellular expression of HCV proteins following transfection. Cells were first fixed in 4% paraformaldehyde (PFA) for 15 minutes at RT before being permeabilized with 100% methanol at -20°C for 20 minutes. Subsequently cells were washed 3 times with PBS, blocked at 37°C for 2 hours in blocking buffer (PBS containing 1% bovine serum albumin (BSA)) and incubated at 37°C for 1 hour with primary antibody (see section 2.1.5.1) diluted appropriately in the blocking buffer. Cells were then washed 3 times with PBS and stained with the appropriate fluorophore-conjugated secondary

antibody (directed against the primary antibody, see section 2.1.5.2) for 1 hour at 37°C in the dark. Finally, cells were washed 3 times with PBS and then stained with DAPI (4',6'-diamidino-2-phenylindole; Life Technologies). The cells were visualized by fluorescence microscopy (Zeiss LSM-700) and digitised using the Zen software (Zeiss).

2.2.3.5 GFP Protein Expression Visualisation

GFP-positive cells were visualised on an Eclipse TE2000 inverted microscope (Nikon) and digitised on the NIS Elements imaging software (Nikon).

2.2.3.6 Flow Cytometry for protein detection

The mean fluorescence intensity of GFP expression was examined by flow cytometry of HEK293T transfected cells. The cells (1×10^6) were harvesting by pipetting, transferred to a 5 ml round bottom tube (BD Biosciences) and pelleted by centrifugation at 1500 rpm for 5 minutes. The cells were resuspended in 100µL flow cytometry buffer (2% v/v FCS, 0.05% wt/v sodium azide, 2mM EDTA in PBS) and analysed on a BD FACSCanto II system (BD Biosceinces) using the forward and side scatter gate to remove cell debris and doublets. The cells were then analysed for the mean fluorescence intensity using the FACSDiva software (BD Biosciences) and results calculated using FlowJo software (Treestar Incorporated).

2.2.3.7 Lactate dehydrogenase (LDH) release assay

LDH activity assay was performed on HEK293T cells using the Pierce™ LDH Cytotoxicity Assay Kit (ThermoFisher Scientific; Catalogue number: 88953) according to the manufacturer's protocol. Cells were cultured in 96-well plates at 1×10^5 cells/well and grown to a confluence of 70–80%. The cells were then transfected with the DNA constructs. As a control cells were treated with Doxorubicin. Following treatment, the LDH cytotoxicity assay was performed according to the manufacturer's instructions. Briefly, 100µl cell culture supernatant was transferred from each well to a 96-well plate and 100µl freshly prepared reaction mixture was added to each well. Following 30 minutes incubation at RT in the dark, the absorbance was determined at 490 nm using a FLUOstar OPTIMA plate reader. The quantity of LDH was expressed as a percentage compared with the total quantity of LDH present in cells treated with 2% Triton X-100.

2.2.4 HCV-VLP Based Work

2.2.4.1 Immunoprecipitation of HCV proteins

HEK293T transfected with vaccine constructs were lysed 72 hours post transfection by 3× freeze-thaw cycles (by placing the tube in liquid nitrogen or dry ice to freeze and thawing at 37°C). Cell culture media was also harvested, centrifuged to remove detached cells before filtration through a 0.45µm filter to remove remaining cell debris. The proteins in the clarified

supernatants were concentrated by ultrafiltration through a 70,000-molecular-weight cutoff filter (70K MWCO; Amicon). 500 µl of the cleared supernatant or cell lysate was incubated with 50 µl protein G-agarose (Roche Diagnostic) at 4°C for 3 hours to reduce the background. The supernatant was then incubated with anti-core or anti-E2 antibodies (both diluted 1:200) for 16 hours at 4°C and then with 50 µl of protein G-agarose (Roche Diagnostic) for 4 hours at 4°C with mixing. The beads were washed repeatedly, and the bound proteins were released and denatured by heating for 5 minutes at 95°C in SDS sample buffer. The immunoprecipitated proteins were analysed by electrophoresis on a 12% polyacrylamide gel. After protein transfer to PVDF membranes, the blots were probed with anti-core (diluted 1:200) or anti-E2 (diluted 1:200) antibody followed by horseradish peroxidase-conjugated anti-mouse immunoglobulin antibody (diluted 1:10,000), with subsequent chemiluminescence detection (see section 2.2.3.3).

2.2.5 Immunisation of mice

2.2.5.1 Mice

Six to eight weeks old female Balb/C mice were obtained from the University of Adelaide Laboratory Animal Services. Mice were housed in HEPA-filtered individually vented cages in The Queen Elizabeth Hospital animal house and were used in accordance with the SA Pathology, Women's and Children's Health Network and University of Adelaide animal ethics committee guidelines.

2.2.5.2 DNA vaccine administration

Female 6-8 weeks old Balb/C mice were injected into the dermal layer of the ear (intradermal injection) with 50µg of endotoxin-free DNA in a final volume of 100µl PBS (50µl or 25ng of DNA per ear). For the cocktail DNA vaccines, each group of animals received a total of 20.65 picomoles per dose per animal. The vaccine dosage was determined using 50µg DNA of the smallest construct (encoding HCV antigens) p-tPAtE1 to set the baseline molecular mass for vaccinations. This ensured that each vaccination received at least 50µg total DNA per dose. The molar concentration for each vaccine construct was calculated using the following equation, where the average molecular mass of one base pair = 660 g/mol:

$$\text{Picomoles/µl} = \frac{\text{DNA Concentration(µg/ml)}}{(0.66 \times \text{DNA Size(bp)})}$$

The vaccinations were administered at 2 or 3 weekly intervals. All interventions were performed under isofluorane anaesthesia to ensure the mice were immobile to allow accurate vaccinations. The mice were weighed and monitored visually following all vaccinations.

2.2.5.3 Tissue collection

2.2.5.3a Blood samples

Blood samples were collected from the facial vein of a mouse. Approximately 100µL of blood was taken per mouse per time point. Serum was isolated from the blood by allowing the blood to clot at RT for 30 minutes followed by centrifugation at 13000 rpm for 30 minutes. The serum was collected and stored at -20 °C until required.

2.2.5.3b Spleen collection

Following the last bleed, the mice were culled and spleens were removed and homogenized using a 70µm cell strainer (BD Falcon) to generate single cell suspensions. Erythrocytes were lysed from splenocytes preparations by incubating the cell suspensions in 2mL of pre-warmed ACK lysis buffer (NH_4Cl 0.15 M, $KHCO_3$ 10 mM, EDTA 0.1 mM in MQH_20, adjusted to pH to 7.2-7.4) at 37°C in 5% CO_2 for 1 minute. 5mL of R10 media (RPMI 1640 media containing 10% FBS, 2 mM L-glutamine, 50 µM β-mercaptoethanol, 1 mM sodium pyruvate, 10 mM HEPES buffer, pen/strep) was added to the suspension to stop the reaction, followed by two washes in R10 media. These samples were assayed immediately after the last wash for T cell responses.

2.2.6 Immunological Assays

Immunological assays were performed to determine the levels of immune responses induced in mice vaccinated with DNA.

2.2.6.1 Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed to determine antibody responses against HCV E1 and E2 proteins. High protein binding 96 well plates were coated with 2µg affinity purified E1E2 proteins (section 2.2.3.1) by incubation overnight at 4°C. The plates were washed with PBS and blocked with 2.5% BSA/PBS for 2 hours at 37°C. The plates were washed with PBS, mouse sera from vaccinated animals, diluted in 2.5% BSA/PBS, were added and the plates were incubated for 4 hours at 37°C or overnight at 4°C. Pre-vaccination mice sera were used as negative controls. The plates were washed with 0.01% Tween 20 in PBS (PBS-T), and a polyclonal HRP-conjugated goat anti-mouse immunoglobulin antibody (Invitrogen) diluted 1: 3,000 in 2.5% BSA/PBS was added. The plates were incubated for 1 hour at 37°C, washed with PBS-T, and developed using 1-Step™ Ultra TMB-ELISA substrate (ThermoFisher Scientific). The plates were left for approximately 5 minutes at RT in the dark, and colour development was then stopped by adding 2.5M HCl. Absorbance at 450 nm was determined on a FLUOstar OPTIMA plate reader (BMG LabTech). Endpoint titres were determined as the reciprocal of the highest

serum sample dilution with an OD reading above the cut-off, set as 2 standard deviations (SD) above the mean OD of serum samples from pre-vaccinated- or naïve- mice.

2.2.6.2 Interferon gamma (IFN-γ) enzyme-linked immunosorbent spot (ELISPOT) assay

The frequency of IFN-γ secreting T cells was measured (section 2.2.5.3b) by performing an ELISPOT Assay. Multiscreen-IP filter plates (Millipore) were coated with anti-IFN-γ antibodies (clone AN18; MabTech, Thomastown, Victoria, Australia) at 5 µg/ml and incubated overnight at 4°C. The plates were washed and blocked with R10 for 2 hours at 37°C. Red blood cell depleted splenocytes were added to duplicate wells at 5×10^5 cells per well and stimulated with 4 µg/ml of either HCV core, E1 or E2 peptides (HCV type 1b J4 Peptides, core catalogue number: NR3737, E1 catalogue number: NR3738, E2 catalogue number: NR3739, provided by BEI Resources, NIAID, Bethesda, MD) for 36 hours at 37°C. The core and E1 peptides consisted of one pool each containing 28 peptides. The E2 peptides were divided into 2 pools containing 28 (peptide 1 to 28) and 27 (peptide 29 to 55) peptides (referred to as E2 pool 1 and E2 pool 2 respectively). The addition of phytohemagglutinin (PHA) represented a positive control and R10 media alone represented the negative control. Anti-mouse IFNγ-biotin (clone R4-6A2; MabTech) was added for 2 hours at RT in the dark. Plates were then incubated with streptavidin-alkaline phosphatase (Sigma Aldrich) for 1 hour at RT in the dark. Spots were formed by the addition of BSigmaFast CIP/NBT (Sigma) and left to develop in the dark for up to 30 minutes. The plates were allowed to dry, and were read on an AID EliSpot Reader (Autoimmun Diagnostika) and analysed using the AID EliSpot software (Autoimmun Diagnostika). The average number of spots (spot forming units; SFU) from the negative control was subtracted from each stimulated sample and the data adjusted to SFU/10^6 splenocytes.

2.2.6.3 Intracellular cytokine staining

Multicolor intracellular cytokine staining (ICS) was performed to analyse immune responses in the spleen (section 2.8.3.1) of vaccinated mice. 4×10^6 cells were incubated with R10 alone or stimulated with 4 µg/ml HCV core, E1 and E2 peptides and incubated with 3 µg/ml protein transport inhibitor (Brefeldin A) or 0.7µl/ml GolgiStop (BD Biosciences) for 12 hours at 37°C. The cells were then washed and stained with surface marker antibodies; CD4-BV510 (eBioscience), CD8-APCe780 and CD44-APC. The cells were then fixed/permeabilised using fluorescence-activated cell sorting (FACS) Cytofix/Cytoperm (BD Biosciences) and stained with the intracellular antibodies; IL-2-PerCP-Cy5.5, TNF-α-PE, IL4-BV421 and IFN-γ-FITC. The cells were resuspended in 5 % FCS/PBS and analysed immediately. After processing, samples were acquired on a BD FACSCanto II flow cytometer and analysed using FlowJo software (Treestar Incorporated). Analysis was performed by subtracting the background in the

unstimulated splenocytes from the frequency in the peptide-stimulated samples. Phytohaemagglutinin (PHA) was used as a positive control for cytokine producing T cells.

Lymphocyte were gated based on their forward scatter-area (FSC-A) and side scatter-area (SSC-A) characteristics. Single events within lymphocytes gate were then gated based on their FSC-A and FSC-H (forward-scatter-height) linear relationship. Single-colour labelled cells were used as control for adjusting compensation for multi-colour analysis. In all cases, positive gates were drawn relative to fluorescence minus one controls (FMO). At least 500,000 events were acquired for each sample.

2.2.6.4 inhibition of binding of HCV VLPs toHuh7 cells by immunized mice sera

The inhibition of binding assay was performed as previously described [420, 421]. Briefly, 50ng HCV-VLPs of genotype 1b were labelled with FITC. The labelled VLPs were pre-incubated with different dilutions of serum (1:50, 1:20 and 1:10) for 1 hour at 37°C and the complex was allowed to bind to Huh 7 cells for 3 hours at RT. Cell-bound fluorescence was analysed using FACS calibur flow cytometer (BectonDickinson) using Winmdi II software to calculate the mean fluorescence intensity (MFI) of the cell population, which directly relates to the surface density of Alexa-labelled HCV-LPs bound to hepatocytes. The MFI values of cells with or without HCV-LPs were compared and percentage binding was determined as per following equation:

$$\%\text{binding of VLP to Huh7 cells} = \frac{\text{experimental MFI} - \text{negative control(only cells) MFI}}{\text{positive control MFI} - \text{negative control(only cells) MFI}} \times 100$$

2.2.6.5 Mapping of antibody epitopes by peptide ELISA.

Epitope mapping was done using a series of overlapping peptides covering the E1E2 region of the HCV isolate J4 sequence. The peptides consisted of 18-mers with an overlap of 11 amino acids covering the E1 or E2 HCV glycoprotein region (BEI Resources, NIAID, Bethesda, MD). Briefly, plates were coated with 5µg/ml of peptide, blocked with 4% BSA/PBS, and incubated with sera for 4 hours and binding was detected in an ELISA format as described above in section 2.2.6.1.

2.2.6.6 HCV competition ELISA

ELISA plates were coated with E1E2 recombinant proteins as described in section 2.2.6.1. Pooled serum from vaccinated mice was diluted (1/50) and then added to the wells and incubated for 1 hour at 37°C. Monoclonal antibodies were then added to the same well at a concentration resulting in 70% maximal binding to E1E2, as determined in prior titration experiments, and incubated at 37°C for 3 hours. Binding of the MAbs was detected with anti-

human HRP-conjugated secondary antibodies. Percentage reduction in absorbance of the monoclonal antibodies was calculated and plotted as % competition.

2.2.7 Statistical analyses

Data presented as mean ± SEM were generated with GraphPad Prism version 6 (GraphPad Software). Statistical significance was determined using the non-parametric Mann-Whitney U test; with a value of $p<0.05$ denoted by *, $p<0.01$ denoted by ** and $p < 0.001$ denoted by ***. considered significant, and $p > 0.05$ was considered non-significant.

Chapter 3. Construction and immunogenicity of DNA vaccines encoding HCV-core, E1, E2 and perforin

3.1 Introduction

Despite the development of potent antiviral drugs, HCV remains a major health problem as approximately 200 million people worldwide are persistently infected [80]. Although the virus persists in a majority of patients, at least 20-30% of patients clear the virus spontaneously, suggesting that immunity to HCV can develop and that the development of a vaccine may be an achievable goal.

Most vaccines currently approved for use in humans rely on the induction of NAbs to prevent or limit infection. Recent studies have suggested that NAbs play an important role in controlling HCV infection as induction of NAb correlate with HCV clearance during infection [129, 422]. NAbs typically recognize tertiary and quaternary structures [423] and therefore a vaccine platform aimed at inducing NAbs require expression of the target proteins, often envelope proteins, in their correct conformation. Several approaches to HCV vaccine development to elicit NAb have been studied and include DNA vaccines. In recent years, novel DNA-based vaccine formulations, which include expression of virus-like particles (VLPs), have also generated promising outcomes. VLPs are attractive candidate to elicit NAbs because they structurally resemble the wildtype virus from which they are derived but are much safer. They offer the opportunity to display envelope proteins in their native surface conformation. VLPs displaying HCV envelope proteins have been reported to induce cross NAb in an animal model [146]. Furthermore, immunisation with plasmid DNA-forming (plasmo-retro) VLPs pseudotyped with HCV E1 and E2 proteins has been shown to induce both humoral and cellular immune responses in mice [424].

Despite the many advantages associated with the use of DNA vaccines, to date no DNA vaccines are approved for use in humans, mainly attributed to the lack of potent immunogenicity in humans [266]. Various forms of adjuvant have been used to increase the immunogenicity of vaccines in general and the use of a genetically encoded adjuvant might be beneficial in a DNA vaccine strategy. Following necrotic cell death, activation of the innate immune system induces inflammation in response to DAMPs released by the dying cells and also alerts the adaptive immune system to potential danger. Dendritic cells (DCs) [266] are central to this process as they are able to cross-present exogenous proteins and activate naïve CD8+ T cells. Therefore, inducing necrotic or lytic cell death in antigen-positive cell *in vivo* would result in the expression and/or release of DAMPs and represent an attractive adjuvant candidate for a DNA vaccine. To date only a few studies have investigated the use of this

mechanism to enhance the immune response to vaccines [383-387]. A DNA construct encoding Listeriolysin O fused to SIV gag was used to enhance immunogenicity to the vaccine [409]. Other studies have focused on the use of molecules such as Fas [410], and caspases 2 and 3 [381] to induce cell death. The Gowans laboratory has previously shown that targeted killing of viral antigen-positive cells using DNA vaccines that also encode perforin (PRF), a cytolytic mediator that disrupts cellular membrane to result in cell death, resulted in improved immunogenicity of the vaccines [414, 416-418]. These results suggest that the use of cytolytic proteins represents an effective mechanism to induce inflammation and activate DCs which are crucial for the activation of the adaptive immune response to vaccine antigens [89, 415, 425]. More importantly, this mechanism may be used to induce lytic cell death which would result in the release of intracellular VLPs, thereby, combining the immunogenicity of VLPs with the ease of production of plasmid DNA.

3.2 Aims

The aims of this chapter were to:

1. Construct cytolytic DNA vaccines encoding the HCV structural proteins core, E1 and E2 as well the cytolytic protein, perforin.
2. Examine protein expression and VLP formation from these constructs *in vitro*.
3. Assess the immunogenicity of these vaccine candidates in mice.

3.3 Results

3.3.1 DNA Vaccine formulation

All the DNA vaccines constructs were formulated based on a bicistronic vector (pJ) that was produced by modifying the commercially available plasmid, pVAX1 (Life Technologies). pVAX1 contains a constitutive cytomegalovirus (CMV) promoter, a multiple cloning site [426] and the bovine growth hormone (BGH) polyadenylation site. To produce the bicistronic pJ vector, pVAX1 was modified to include a second promoter from Simian Virus 40 (SV40), a novel MCS and the SV40 polyadenylation site (SV40 pA) [416, 425]. The genes encoding the HCV proteins core, E1 and E2 were cloned under the control of the CMV promoter while the PRF gene was inserted downstream of the SV40 promoter, which is estimated to be 10-fold weaker in terms of driving gene expression compared to the CMV promoter [415, 427]. This strategy (Figure 3.1) was designed to express reduced levels of PRF relative to HCV structural proteins expressed from the CMV promoter and therefore provide an optimum combination of antigen and cytolytic protein expression, as intracellular VLPs resulting from expression of core, E1 and E2 are expected to be produced before the threshold of PRF expression required for cell lysis, and thus VLP release, is reached.

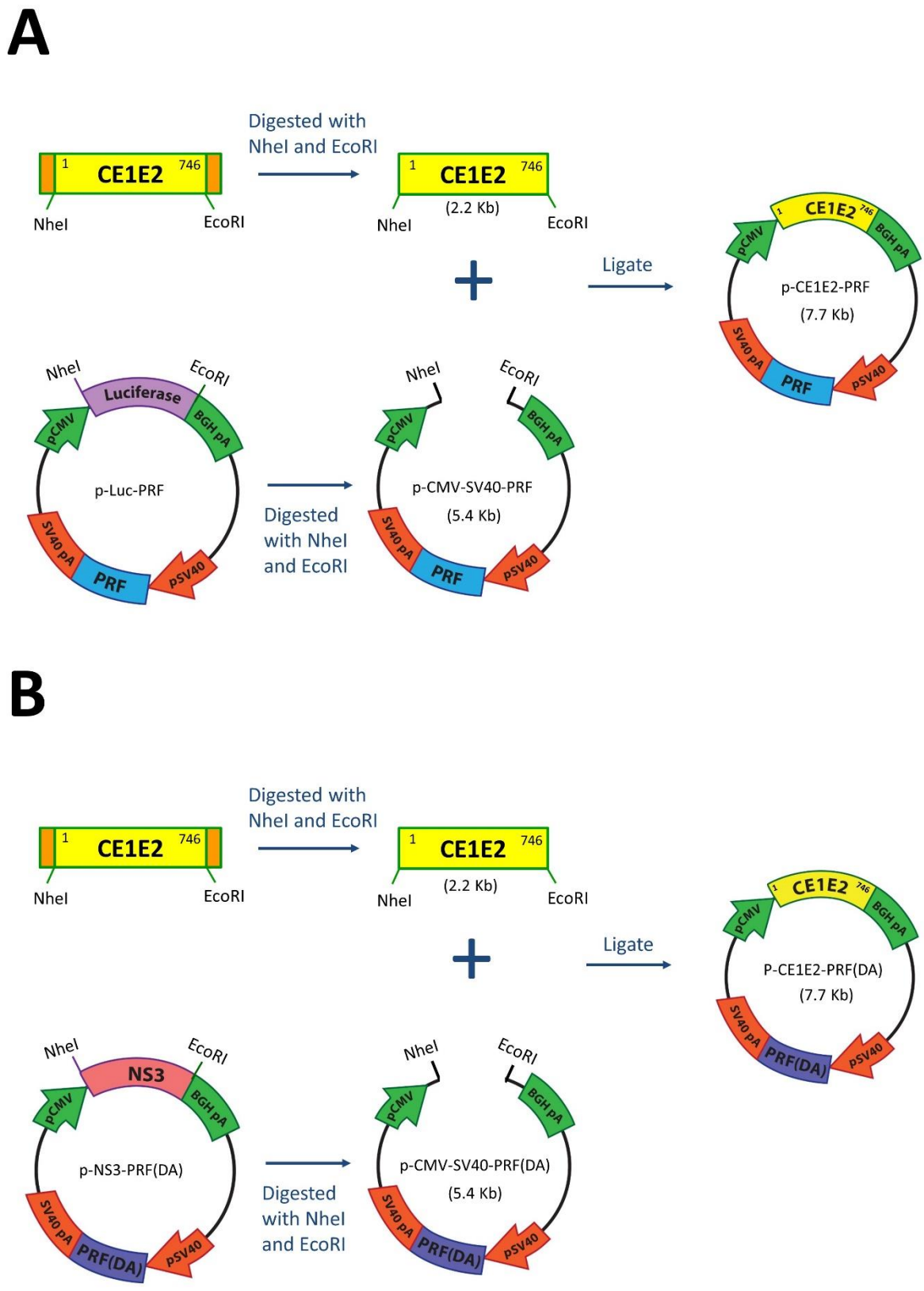


Figure 3.1. The strategy used to insert the HCV-CE1E2 gene downstream of the CMV promoter to produce (A) p-CE1E2-PRF and (B) p-CE1E2-PRF(DA). The numbering corresponds to the amino acid position in the polyprotein of the genotype 1 HCV strain. DNA plasmid sizes are shown in brackets.

3.3.2 Production of the DNA vaccine constructs

For effective target cell death, a truncated version of PRF with a 12 amino acid deletion at the C terminus (referred to as PRF in this chapter for simplicity) was used [428]. This mutation resulted in PRF retention in the ER and the induction of cell death. PRF is required to bind to calcium in order to oligomerize and form pores in the cellular membrane, resulting in cell lysis. A D483A mutation in the key calcium binding residue has been shown to result in loss of PRF cytolytic ability by preventing PRF pore formation and therefore allow the cell to survive [89, 428]. Consequently, as controls for PRF-induced cell lysis, constructs encoding this mutant version of PRF, referred to in this chapter as PRF(DA), were generated.

3.3.2.1 Construction of p-CE1E2-PRF and p-CE1E2-PRF(DA)

Codon-optimised genes encoding the HCV structural proteins (GenBank accession number AF139594.2) were generated by subjecting the sequence to the GeneOptimizer bioinformatic algorithm, followed by synthesis of the codon-optimized genes (GeneArt, Regensburg, Germany). The genes were codon optimised for expression in mammalian cells, contained a Kozak sequence, and start and stop codons. The synthesised genes were used as template to generate DNA products encoding CE1E2 by PCR, using primers Core fwd and E2 rev (appendix I) containing the NheI and EcoRI restriction sites respectively. The PCR products were analysed by gel electrophoresis to detect the amplified fragments (Fig. 3.2A). A plasmid encoding Luciferase and PRF (p-Luc-PRF – Fig. 3.1A) was similarly digested to facilitate ligation. The ~5.5kb band corresponding to p-CMV-SV40-PRF or p-CMV-SV40-PRF(DA) was excised from the gel and the DNA was extracted as described in Chapter 2 – section 2.2.2.6, followed by ligation and transformation into *E. coli* (DH5α) cells. Positive clones were identified by colony PCR using the primer pair Core-fwd and E2-rev (Fig. 3.2C) and further analysed in a series of restriction enzyme digestion studies (Fig. 3.2D). The orientation of the CE1E2 genes was not a concern since two different restriction enzyme sites were used for cloning (directional cloning). The correct sequences of the positive clones were confirmed by DNA sequencing. Constructs encoding the core protein alone or the E1E2 envelope proteins as a single polyprotein were produced to assess the formation of VLP by p-CMV-SV40-PRF, as these proteins are not expected to self-assemble into VLPs when expressed separately [429]. These control constructs also encoded either PRF or PRF(DA). The strategy used to produce these constructs is described in chapter 2 – section 2.2.2. A complete catalogue of plasmid maps is shown in Figure 3.3.

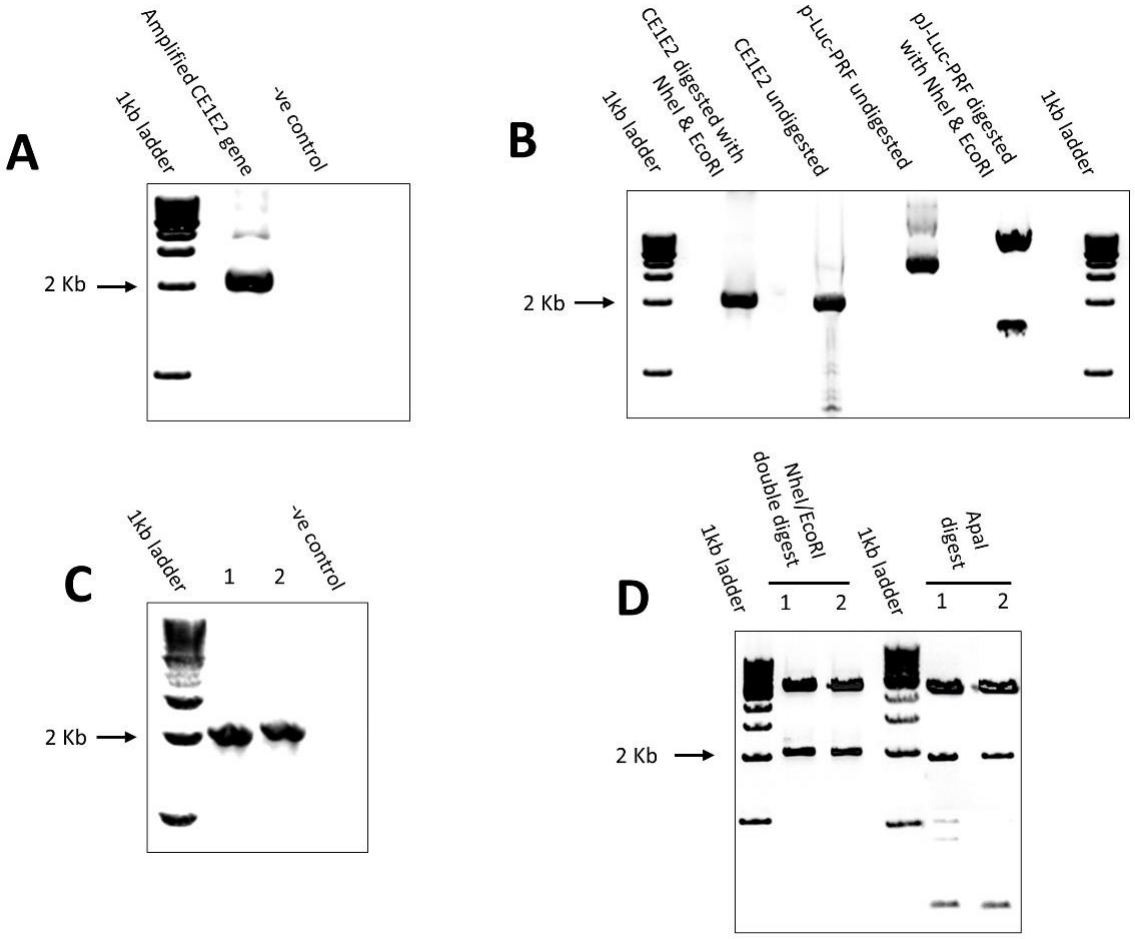


Figure 3.2. The production of p-CMV-CE1E2-SV40-PRF. A) Gel electrophoresis analysis of the HCV-CE1E2 gene amplified by PCR. B) The digestion of HCV-CE1E2 and p-Luc-PRF with NheI and EcoRI restriction enzymes. C) Random colonies, lane 1 and 2, were picked from agar plates and colony PCR was performed to identify positive clones. The colony PCR products were analysed by gel electrophoresis analysis on a 0.8% agarose gel. D) Restriction enzyme digestion of positive clones performed with NheI, EcoRI and ApaI analysed by gel electrophoresis.

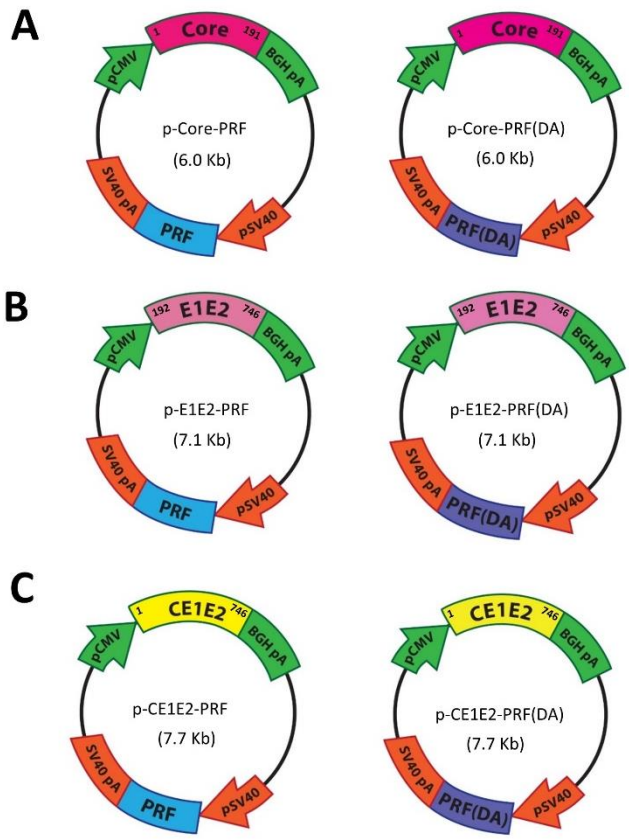


Figure 3.3. Plasmid maps of DNA constructs used in this chapter. A) DNA plasmids produced by inserting core into the pJ-SV40-PRF or pJ-SV40-PRF(DA) constructs. B) DNA vaccine constructs produced by inserting E1E2 into the pJ-SV40-PRF or pJ-SV40-PRF(DA) constructs. C) DNA plasmids encoding HCV-CE1E2 and PRF or PRF(DA) generated to compare the levels of VLP formation and release. The numbering corresponds to the amino acid position in the HCV polyprotein. DNA plasmid sizes are shown in brackets.

3.3.3 HCV structural protein expression from DNA vaccine constructs

Prior to vaccination studies, it was necessary to confirm protein expression from the DNA constructs described above. Each construct was transfected into HEK293T before analysis by western blot or immunofluorescence as stated in chapter 2 – section 2.2.3.

3.3.3.1 Detection of HCV structural proteins

SDS-PAGE analysis of lysates of HEK293T cells followed by immunoblot analysis (western blot) was performed to detect HCV-core, E1 or E2 proteins expressed in cells transfected with the DNA constructs p-Core-PRF, p-Core-PRF(DA), p-E1E2-PRF p-E1E2-PRF(DA), p-CE1E2-PRF or p-CE1E2-PRF(DA).

Immunoblotting with anti-core antibodies revealed an expected band of approximately 21 kDa in lysates of cells transfected with p-CE1E2-PRF, p-CE1E2-PRF(DA), p-Core-PRF, p-Core-PRF(DA) (Fig. 3.4A). No bands were detected in cell lysates of untransfected cells, cells transfected with pVax, p-E1E2-PRF or p- E1E2-PRF(DA) (Fig. 3.4A). Equal loading was shown using the β-actin loading control.

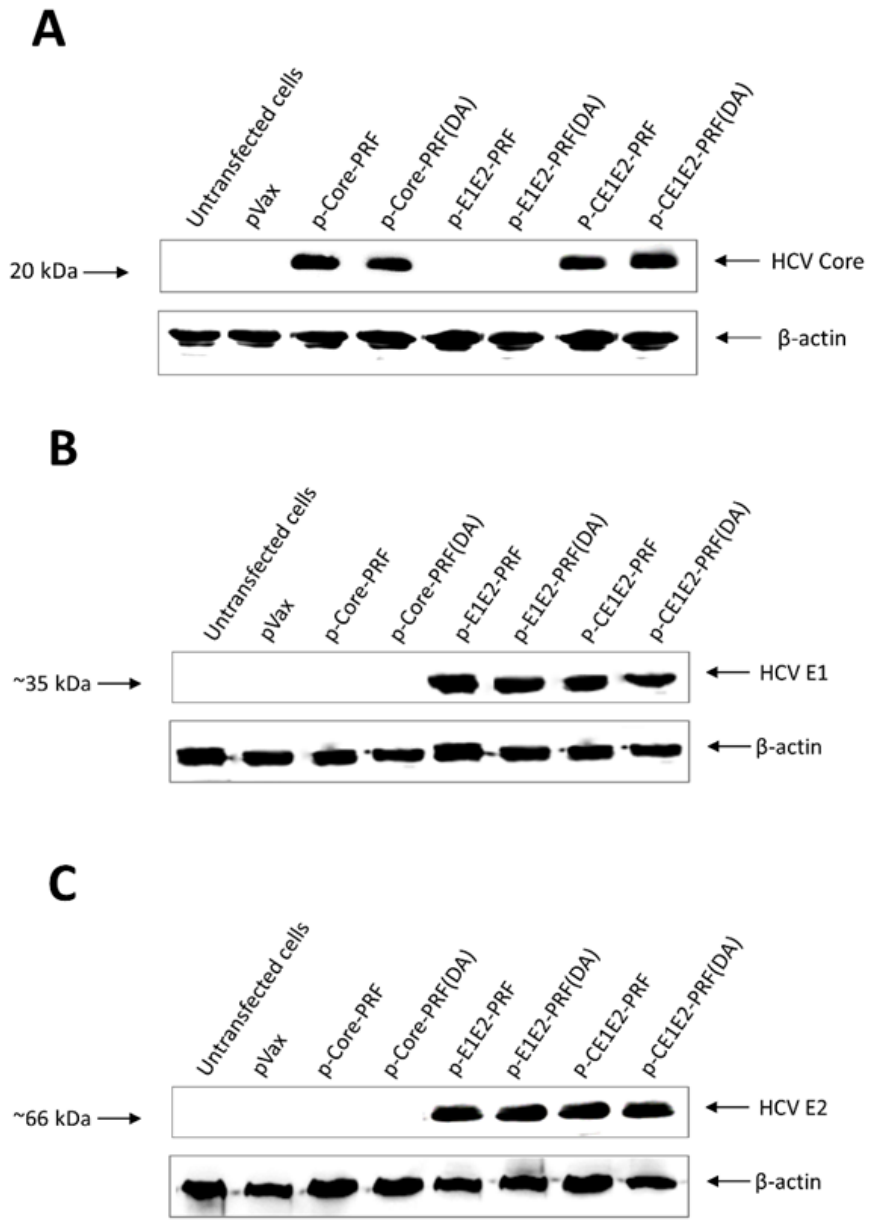


Figure 3.4. Expression of HCV structural proteins from the DNA vaccines. HEK293T cells were transfected with p-Core-PRF, p-Core-PRF(DA), p-E1E2-PRF, p-E1E2-PRF(DA), p-CE1E2-PRF or p-CE1E2-PRF(DA). At 72 hours post transfection, the cells were lysed as described in chapter 2 – section 2.2.3. The cell lysates were then subjected to SDS-PAGE and immunoblotting (IB) with anti-core (A) anti-E1 (B) or anti-E2 (C) antibodies. Molecular masses (in kilodaltons) of protein molecular weight (MW) markers are indicated on the left; HCV-specific proteins are indicated on the right. The untransfected cells and pVax-transfected cells represent controls for non-specific bands, and the β-actin acted as a loading control.

Cell lysates were also analysed for expression of E1 (Fig. 3.4B) and E2 (Fig. 3.4C). The blots stained with anti-HCV E1 showed a band of ~35kD corresponding to E1 (Fig. 3.4B), while anti-HCV E2 revealed a band of ~66kD corresponding to E2 (Fig. 3.4C), in the lysate of cells transfected with p-CE1E2-PRF, p-CE1E2-PRF(DA), p-E1E2-PRF or p-E1E2-PRF(DA). No bands corresponding to E1 or E2 were detected in samples from untransfected cell controls, or pVax, p-Core-PRF or p-Core-PRF(DA) transfected cells (Fig. 3.4B).

For completeness, the expression of the HCV structural proteins in HEK293T cells transfected with the DNA vaccine constructs was also demonstrated by immunofluorescence analysis using anti-HCV core, or E1/E2 antibodies in addition to anti-HCV serum from HCV-infected patients (Fig. 3.5 and Fig. 3.6) as described in Chapter 2 – section 2.2.3.4. After staining with anti-core antibodies, fluorescence staining was observed in cells transfected with p-Core-PRF, p- Core-PRF(DA), p-CE1E2-PRF or p-CE1E2-PRF(DA) (Fig. 3.5). No core-specific fluorescence staining was detected in cells transfected with pVax, or in cells transfected with pVAX-GFP (results not shown), pVax, p-E1E2-PRF or p-E1E2-PRF(DA).

HEK293T cells were stained with anti-HCV-E1 or anti-HCV-E2 antibodies and immunofluorescence staining was observed in the cells transfected with p-E1E2-PRF, p-E1E2-PRF(DA), p-CE1E2-PRF or p-CE1E2-PRF(DA). No fluorescence was observed in cells transfected with pVax, p-Core-PRF, p-Core-PRF(DA) (Fig. 3.5 – middle and right panels) as expected. The immunostaining analysis also revealed that the expression of HCV structural proteins was mostly confined to the cytoplasm and plasma membrane.

3.3.4 Expression of perforin from vaccine constructs

3.3.4.1 Perforin expression

Each of the DNA constructs described above encoded either PRF or the control PRF(DA) in addition to the immunogen and therefore it was necessary to confirm PRF expression. HEK293T cells were transfected with the DNA constructs and incubated for 48 hours post transfection. PRF expression was detected by immunofluorescence as described in Chapter 2 – section 2.2.3.4, using anti-PRF antibodies and anti-HCV (pooled HCV genotype 1b patient sera). The results of this experiment showed that PRF was expressed in the cells transfected with DNA construct encoding PRF or PRF(DA) and more importantly in cells transfected with the vaccine constructs p-CE1E2-PRF and p-CE1E2-PRF(DA) (Fig. 3.6) and in cells transfected with a construct in which PRF expression was controlled by the stronger CMV promoter (p-CMV-PRF). This not only served as a positive control for PRF expression (Fig. 3.6) but also as a positive control for PRF-induced cell lysis (Fig. 3.7). PRF expression was not observed in cells transfected with pVax or the untransfected cell controls (data not shown). As expected the

levels of PRF expression (evident by fluorescence intensity) were higher in cells transfected with p-CMV-PRF. The results also showed that the expression of PRF correlated with the expression of the HCV proteins (Fig. 3.6), confirming that these are co-expressed with PRF in individual cells following transfection with the vaccine constructs.

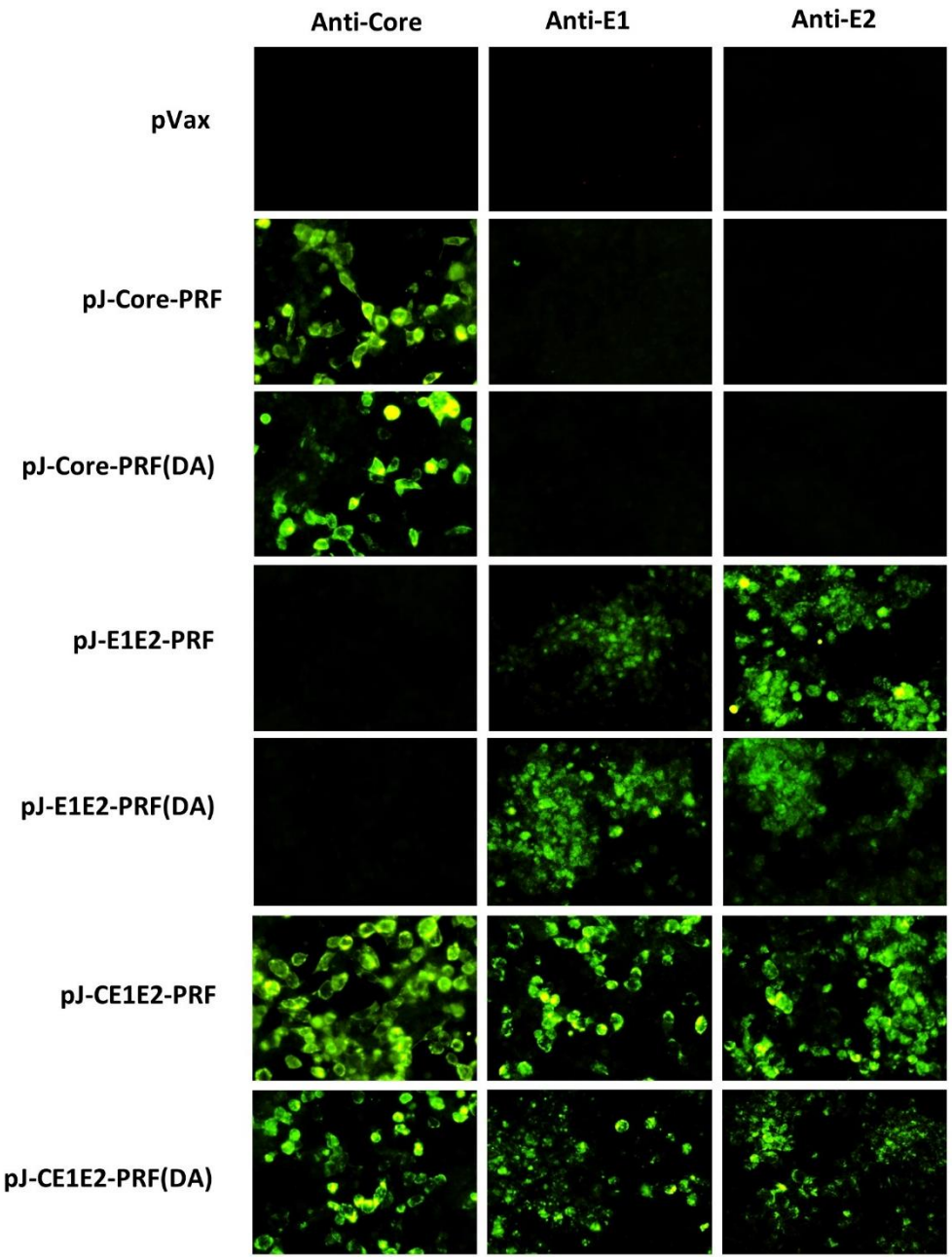


Figure 3.5. Immunofluorescence of HCV structural proteins expressed in HEK293T cells. Fluorescence micrograph after transfection of HEK293T cells with p-Core-PRF, p-Core-PRF(DA), p-E1E2-PRF, p-E1E2-PRF(DA), p-CE1E2-PRF or p-CE1E2-PRF(DA). At 48 hours post-transfection, the cells were fixed and processed as described in chapter 2 – section 2.2.3.4. Protein expression was analysed with anti-core, anti-E1 or anti-E2 antibodies. Cells transfected with pVax served as controls for non-specific fluorescence (magnification: 400×).

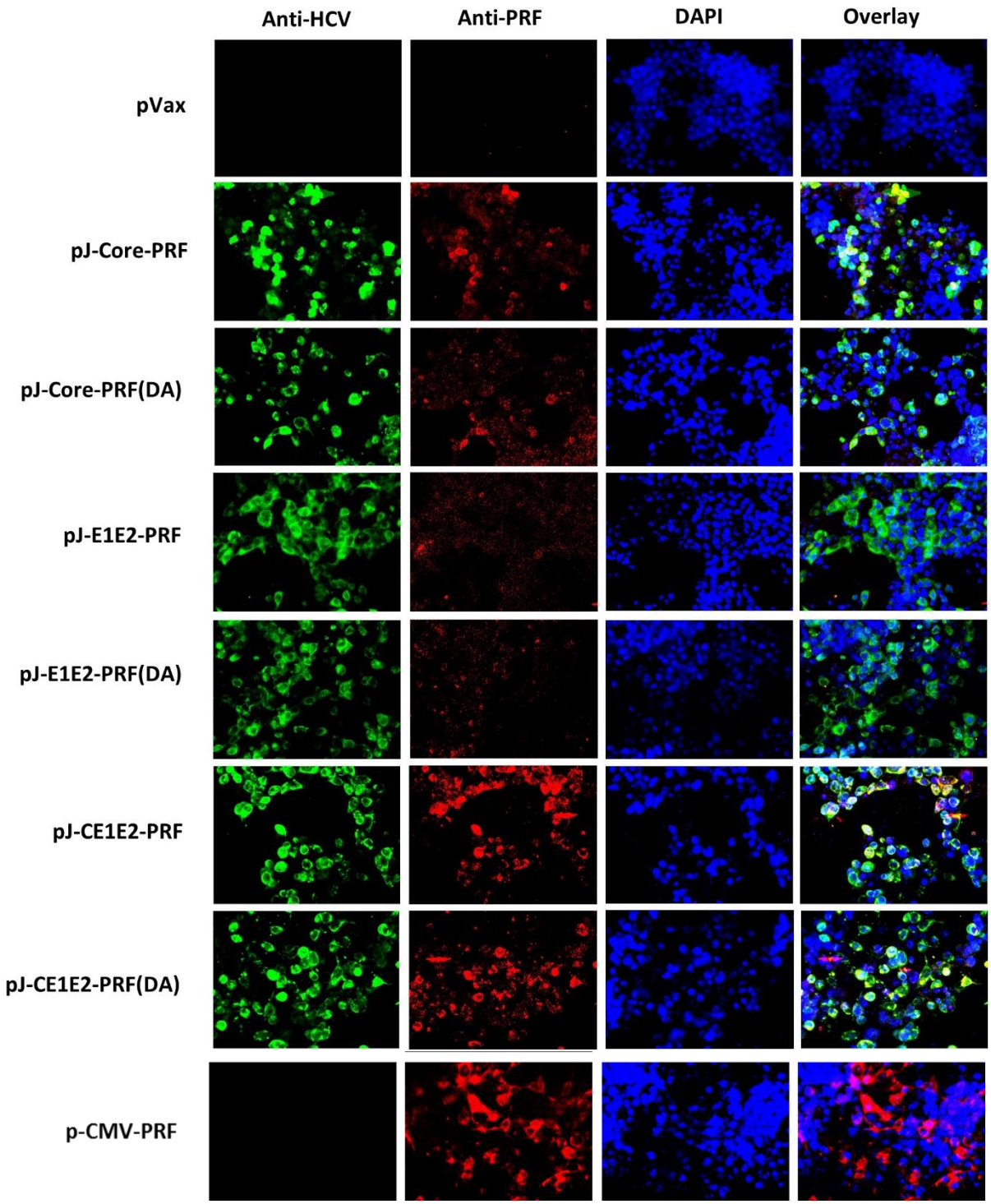


Figure 3.6. Immunofluorescence of HCV structural proteins and perforin expression in HEK293T cells. Fluorescence micrograph after transfection of HEK293T cells with pVax, pJ-Core-PRF, pJ-Core-PRF(DA), pJ-E1E2-PRF, pJ-E1E2-PRF(DA), pJ-CE1E2-PRF, pJ-CE1E2-PRF(DA) or p-CMV-PRF(DA). 72 hours post transfection, the cells were fixed/permeabilised and probed with anti-HCV (pooled HCV genotype1 patient sera, green), anti-PRF (red) and DAPI staining for nuclei (blue). p-CMV-PRF(DA) served as positive control for PRF expression and cells transfected with pVax were used as background control. Fluorescence was observed using a ZEISS LSM 700 microscope (magnification: 400×).

3.3.4.2 Assessment of perforin cytolytic activity

The cytolytic activity of PRF was investigated by monitoring lactate dehydrogenase [430] [430] released from cells transfected with the vaccine constructs. In this assay, the LDH remains intracellular in viable or apoptotic cells but is released into the extracellular milieu if the cell dies in a lytic or necrotic manner. LDH was assayed as described in chapter 2 – section 2.2.3.7.

Cells transfected with the control p-CMV-PRF released a significantly higher (44.8% relative to maximum release control) level of LDH compared to the apoptosis-inducing doxorubicin (4.5%) (Fig. 3.7). p-CE1E2-PRF transfection resulted in the release of LDH similar to that of the positive control (36.6%). Cells transfected with p-CE1E2-PRF(DA) did not result in significant LDH release above the negative controls pVax (16.2% and 16.5% respectively – Fig. 3.7). Collectively, these results demonstrate that PRF expression results in cell membrane damage and release of LDH, while the expression of PRF(DA) is devoid of such cytoxicity.

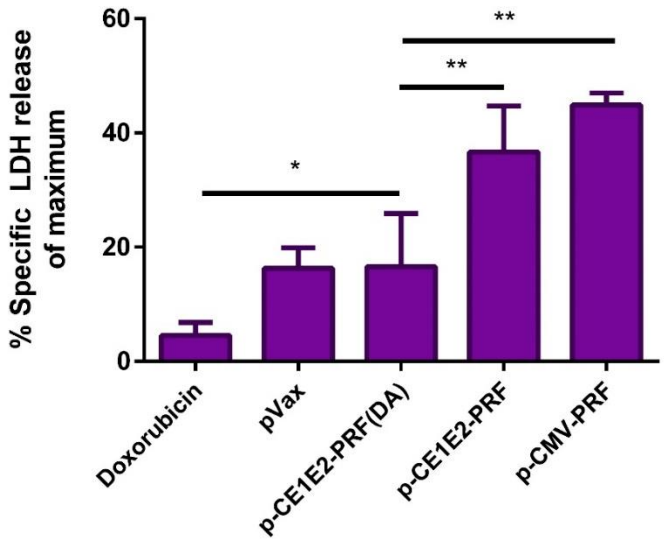


Figure 3.7. LDH release following transfection of HEK293T cells with VLP vaccine constructs. HEK293T cells were transfected with VLP vaccine constructs and 72hr P.T the supernatant was collected and LDH release was analysed. Cells transfected with p-CMV-PRF served as positive control for PRF lytic activity. Cells treated with Doxorubicin which induces apoptosis served as negative controls for LDH release. The percentage cytotoxicity was calculated by subtracting the LDH activity of the untreated normal cells (Spontaneous LDH Release) control from the LDH activity of transfected cells, divided by the total LDH activity [(maximum LDH release control activity) – (spontaneous LDH release control activity)], and multiplied by 100. Data pooled from 2 independent experiments showing the mean (n = 6) ± SEM. Mann-Whitney non-parametric t-test was performed where appropriate. Significance tested against p-CE1E2-PRF(DA) control. * $P \leq 0.05$, ** $P \leq 0.01$.

3.3.5 HCV-VLP formation from DNA constructs

3.3.5.1 Co-immunoprecipitation of Core and E2 proteins

VLPs are typically purified using methods such as sucrose or caesium chloride gradient ultracentrifugation [431]. In this chapter, the expression of VLPs was assessed as described previously using iodixanol gradient ultracentrifugation [420, 421] (chapter 2 – section 2.2.4.2). Briefly, HEK293T were transfected with p-CE1E2-PRF or p-CE1E2-PRF(DA) as these constructs are expected to result in the expression and formation of VLPs. 48 hours post transfection, the cell culture medium was collected and concentrated while the cells were lysed using the freeze-thaw method. The concentrated cell culture medium or cell lysis supernatant were layered on to a 10 – 40% continuous iodixanol gradient and centrifuged at 16^{o} C for 16 h at 25 000 rpm in an SW40 rotor followed by the collection of 12 fractions. However, proteins were present at low concentrations in each of the collected fractions, which made their recovery difficult, subsequently, hampering the assessment of formed particles by western immunoblot analysis.

Alternatively, to determine whether the HCV proteins expressed from the vaccine constructs were capable of assembling into VLPs, the association of the core and E2 proteins was investigated. HEK293T cells were transfected with p-CE1E2-PRF or p-CE1E2-PRF(DA) and lysed using the freeze-thaw method to allow assessment of VLP formation from both constructs as PRF(DA) does not induce cell death. The cell culture medium was also collected and concentrated. The protein in the lysates (Fig. 3A & B) and cell culture medium (Fig. 3C & D) was subjected to immunoprecipitation (IP) with anti-E2, anti-core antibodies or normal human serum (NHS). The immunoprecipitated proteins were in turn subjected to SDS-PAGE and immunoblotting (IB) with anti-core or anti-E2 antibodies. Bands of approximately 50 kDa and 26 kDa in size were detected during IB, corresponding, respectively, to the heavy (HC) and light (LC) IgG chains of the antibodies used for immunoprecipitation. Immunoprecipitation with anti-E2 resulted in the detection of core and E2 in the immunoprecipitated complexes when probed with anti-core (Fig. 3.8A & C, left panels) or anti-E2 (Fig. 3.8B & D, left panels) antibodies respectively, consistent with immunoprecipation of a VLP. Bands from concentrated supernatants generally showed a weaker signal compared to cell lysates due to low protein recovery. As expected, core and E2 bands were more pronounced in immunoprecipitated samples from the supernatant of p-CE1E2-PRF transfected cells compared to p-CE1E2-PRF(DA) (Fig 3.8C &D – left panels). Only a faint band corresponding to the core protein was detected when anti-core immunoprecipitated complexes were probed with anti-core antibodies (Fig. 3.8A, middle panel). This was possibly a result of free core protein that is detectable in the supernatant and not part of VLP. No bands specific to either core or E2 were detected in

anti-core immunoprecipitated samples probed with anti-E2 (Fig. 3.8B & D, middle panels) or in samples immunoprecipitated with normal human serum (Fig. 3.8A-D, right panels). These results revealed the coimmunoprecipitation of core and E2 with anti-E2 consistent with the formation of VLPs in HEK293T cells.

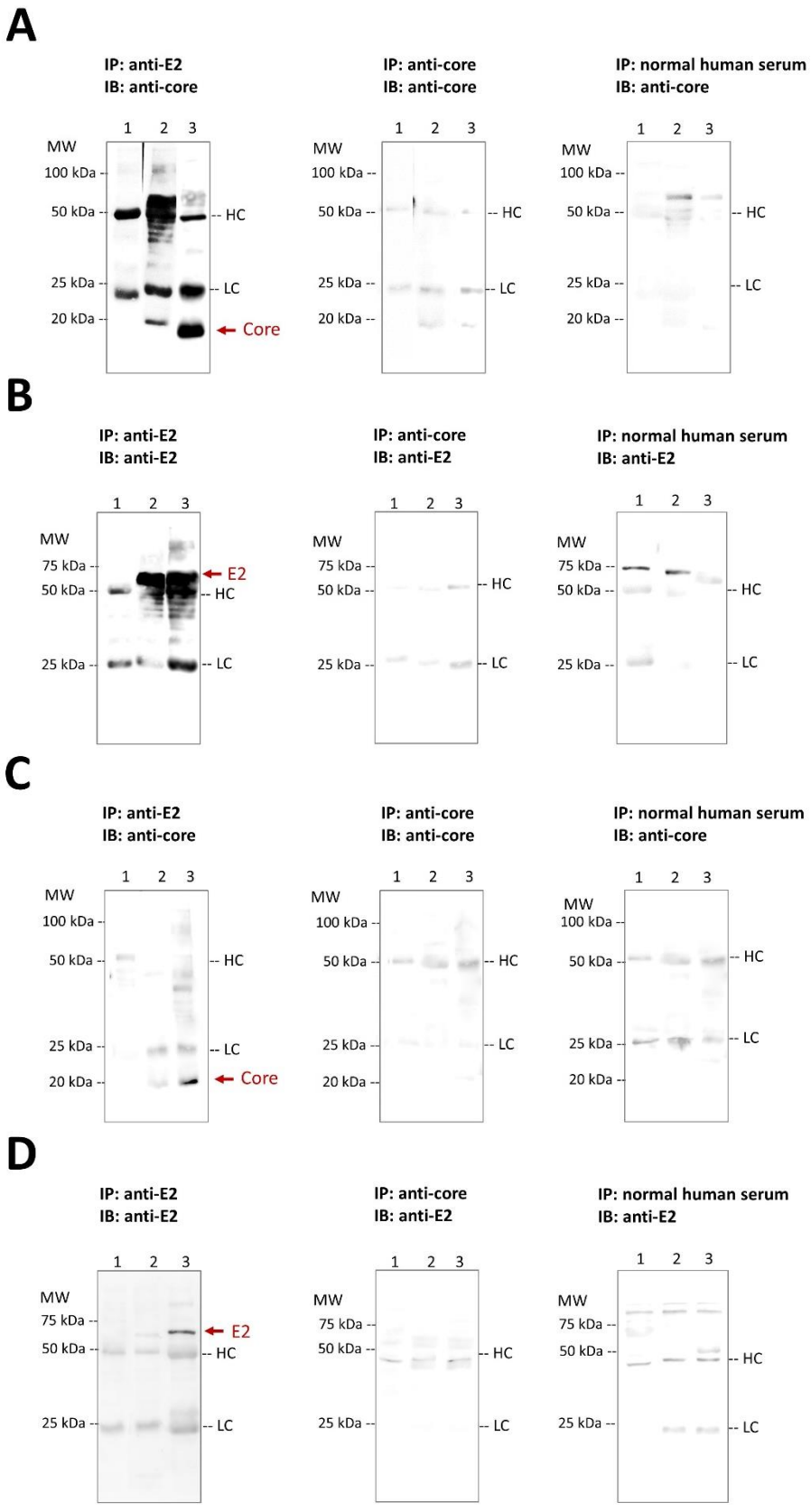


Figure 3.8. Coimmunoprecipitation of HCV structural proteins in HEK293T cells. HEK293T cells were transfected with (lane 2) p-CE1E2-PRF(DA) or (lane 3) p-CE1E2-SV40-PRF. 36 hours post-transfection, the cells were lysed and the cell-culture media was collected and concentrated by ultrafiltration through a 70,000-molecular-weight cutoff filter (70K MWCO; Amicon). The (A&B) cell lysates and (C&D) supernatant samples were subjected to immunoprecipitation (IP) with an anti-core, anti-E2 antibodies or normal human serum as indicated. The immunoprecipitated proteins were subjected to SDS-PAGE and immunoblotted (IB) with anti-core (A&C) or anti-E2 antibodies (B&D) as indicated. Untransfected cells (lane 1) were used as control for non-specific banding. Molecular masses (in kilodaltons) of protein molecular weight (MW) markers are indicated on the left; HCV-specific proteins are indicated on the right in red. The heavy chain (HC) and light chain (LC) of the antibodies used for immunoprecipitation are indicated on the right.

3.3.6 Immunisation of mice with cytolytic DNA vaccine

3.3.6.1 Vaccination schedule

In order to evaluate the potential efficacy of the p-CE1E2-PRF and p-CE1E2-PRF(DA) plasmids as DNA vaccines, BALB/c mice were injected intradermally (in the ear pinnae) with 50µg of the respective plasmid, followed by 5 booster injections at 2 weekly intervals (Fig. 3.9). Blood samples were collected (retro-orbital bleed) two days prior to each vaccination and spleens and blood were harvested 4 weeks after the last vaccination.

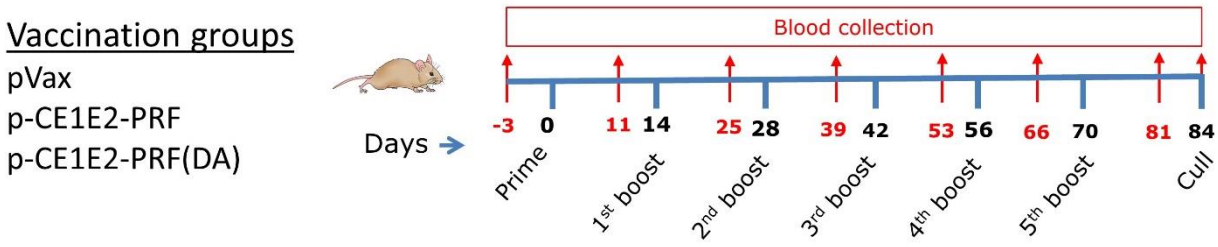


Figure 3.9. The DNA vaccination regimen in 6-8 weeks old female Balb/C mice. The mice were vaccinated six times at two weekly intervals with 50µg DNA. Blood was collected prior to each vaccination and spleens were harvested 14 days post final vaccination.

3.3.6.2 Induction of HCV specific humoral response by CE1E2-PRF

3.3.6.2.1 Purification of HCV-E1E2 protein for use in ELISA

The HCV envelope proteins were expressed in HEK293T following transfection with the p-tPAtE1tE2-histag construct (Fig. 3.10A), purified then used to coat ELISA plates to evaluate the levels of antibody titres in vaccinated mice. The development of the p-tPAtE1tE2 construct and the expression and purification of the 6×His-tagged E1E2 protein is described in more

detail in Chapter 2 - section 2.2.3.1, and chapter 4 – section 4.3.1. To confirm the identity of the purified recombinant protein, a western blot was performed using anti-HCV (genotype 1b HCV-positive human serum). The results clearly demonstrated a band at ~60 kDa in elution 1 and 2 from the nickel column but not in the flowthrough (Fig. 3.10B). Therefore, this ~60 kDa protein was used for coating ELISA plates to evaluate E1 and E2-specific antibodies.

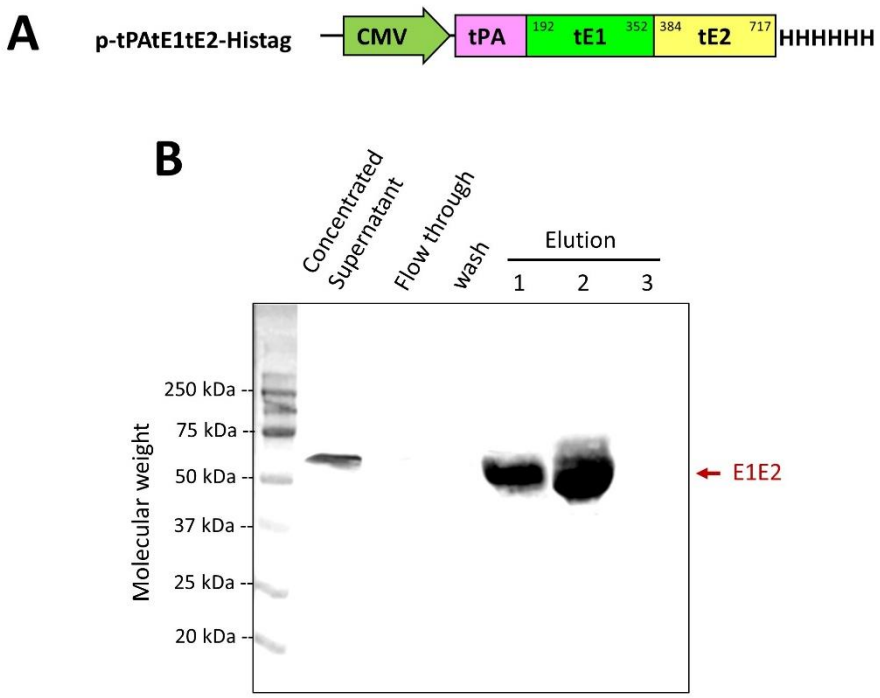


Figure 3.10. Expression and purification of secreted HCV-E1E2 protein determined by western blot analysis. A) Schematic of DNA construct encoding secreted E1E2 protein showing the location of the CMV promoter, the tPA leader sequence E1 and E2 proteins and the C-terminal poly-His tag. B) HEK293T cells were transfected with p-tPAtE1tE2-Histag. At 48 hours post transfection, cell culture supernatants containing secreted glycoproteins were filtered, concentrated, affinity purified, and then subjected to the SDS-PAGE and western blotting as described in chapter 2 – section 2.2.3.3, before detection with anti-HCV antibody. Markers are shown on the left.

3.3.6.2.2. Detection of HCV-envelope-specific antibodies

The serum levels of anti-E1E2 antibodies were monitored by ELISA following each vaccination and recorded as positive if they were two standard deviations above the mean titre in pVax-vaccinated mice. The results showed that vaccination with p-CE1E2-PRF resulted in the highest anti-E1E2 antibody levels while the antibody response peaked and reached a plateau following the 4th booster vaccination (Fig. 3.11A). The antibody titres were also measured 14 days following the last vaccination. All p-CE1E2-PRF vaccinated mice showed a significant anti-E1E2 response with end-point titres ranging from 1/20 to 1/63.2 (Fig. 3.11B), while p-

CE1E2-PRF(DA) or pVax-vaccinated mice showed no detectable anti-E1E2 response (Fig. 3.11B).

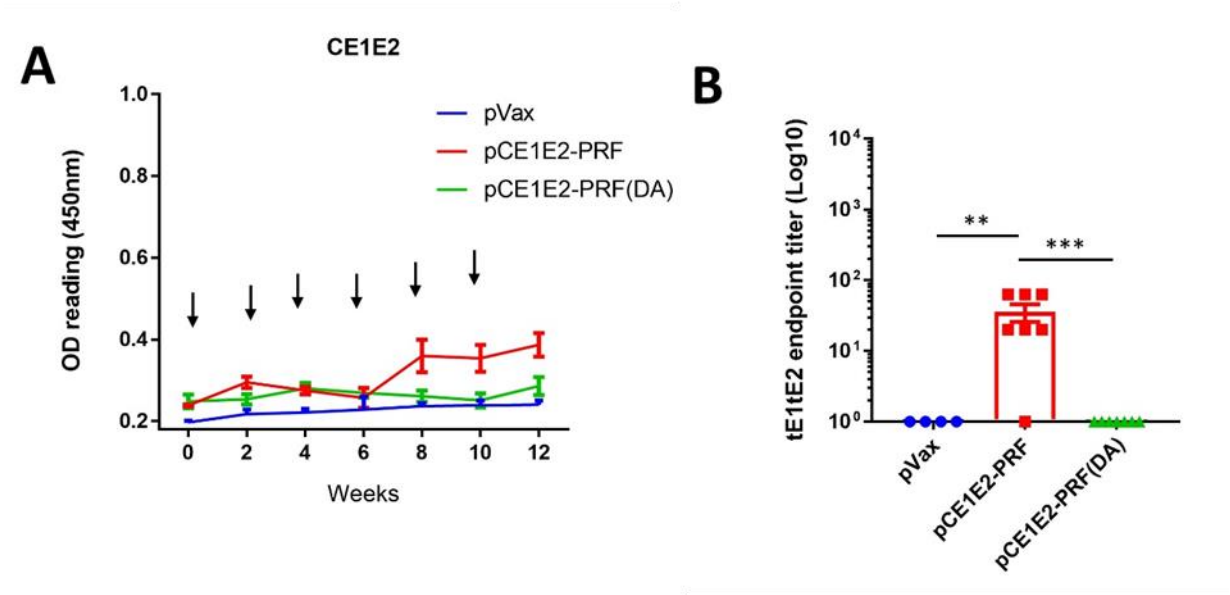


Figure 3.11. Induction of anti-E1 and anti-E2 antibodies by p-CE1E2-PRF and p-CE1E2-PRF(DA) in mice. A) Serum antibody response specific for HCV E1E2 proteins. Serum from vaccinated mice was diluted (1/50) and anti-E1E2-specific antibodies were measured by ELISA at the indicated time points. Values represent mean responses in each group (n = 7) ± SEM. Black arrows represent points when the vaccines were administered. B) Two weeks after the final immunisation, serum from individual mice was serially diluted, and endpoint binding titers for immunised mouse sera were calculated. Values for individual mice are shown (n = 7) and bars represent the mean ± SEM. Mann-Whitney non-parametric t-test was performed to assess significant p-values between the vaccinated groups, **p ≤ 0.01, ***p ≤ 0.001. The data were derived from three independent experiments performed in duplicate. Positive results were determined by values greater than two standard deviations above the mean control of antibody titers detected in pVax-vaccinated mice.

3.3.6.3. Induction of HCV-specific cell mediated response by CE1E2-PRF

The cell-mediated immune response to core, E1 and E2 following vaccination with p-CE1E2-PRF or p-CE1E2-PRF, was initially assessed by ELISPOT to detect IFNγ-secreting cells in the mouse spleen. Splenocytes were harvested from the vaccinated mice two weeks after the last immunization and were stimulated with genotype 1b core, E1 and E2 peptides *in vitro* as stated in chapter 2 section 2.2.6.2. The number of IFN-γ-secreting T-cells was determined by counting spot-forming units (SFUs) in an ELISpot assay.

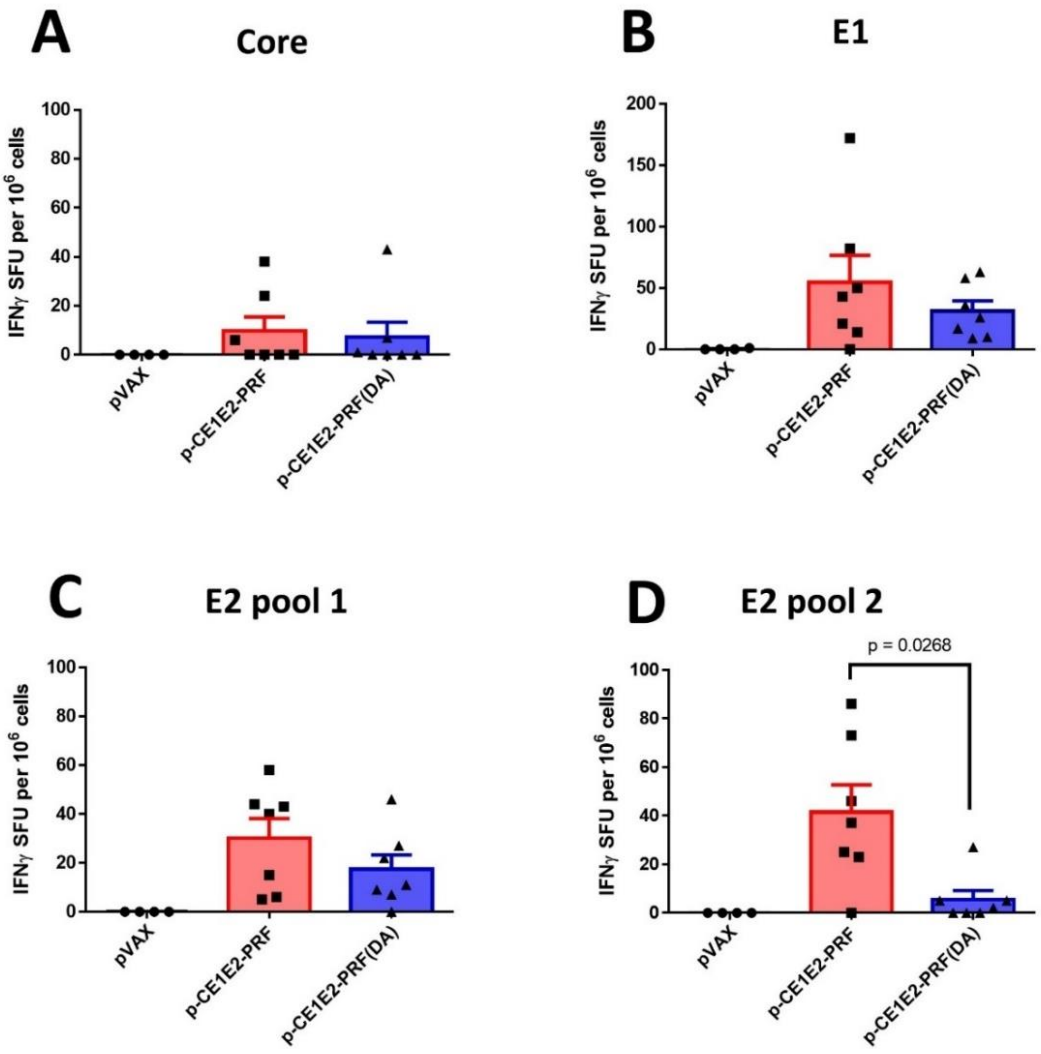


Figure 3.12. HCV-specific IFN-γ production by splenocytes determined by ELISPOT. Mice were vaccinated 7 times at 2 weekly intervals with 50μg doses of DNA. The spleens were harvested 14 days post final vaccination and the splenocytes were stipulated with core, E1 or E2 pool 1 or E2 pool 2 peptide pools. The graphs show IFNγ T cell responses to core (A), E1 (B) and E2 pool 1 (C) and E2 pool 2 (D) in mice vaccinated with pVax, p-CE1E2-PRF or p-CE1E2-PRF(DA). The number of IFNγ secreting T cells was adjusted to spot forming units (SFU) per 10^6 cells. The number of SFU in unstimulated splenocytes was subtracted from the number in peptide-stimulated cells to generate the net HCV response. Data are plotted from 4-7 individual mice per group, showing the mean SFU (±SEM). Mann-Whitney non-parametric t-test was performed. Significance tested against p-CE1E2-PRF(DA) control. Note that the Y-axes may differ

As shown in figure 3.12, although the responses were generally poor, mice vaccinated with p-CE1E2-PRF showed significant numbers of IFN-γ-secreting T-cells (p-value = 0.0268) following stimulation with E2-peptide pool 2 compared to p-CE1E2-PRD(DA)-immunised mice (Fig. 3.12D). Although IFNγ SFUs were consistently higher in p-CE1E2-PRF-vaccinated mice, as opposed to p-CE1E2-PRD(DA)-vaccinated mice, following stimulation with core (Fig. 3.12A), E1 (Fig 3.12B) and E2 peptide pool 1 (Fig 3.12C), these differences were not statistically significant. As expected, the control mice vaccinated with pVax failed to generate any IFNγ-positive cells against each of the peptide pools (Fig. 3.12).

To examine the cell mediated response more quantitatively, ICS was performed on splenocytes from the immunised mice to analyse intracellular cytokine production at the level of a single cell to identify single, double, triple or quadruple cytokine-producing T cells. In chronic HCV infections, such multifunctional T cells have been associated with protective immune responses [432]. Splenocytes isolated from vaccinated mice at week 12 were stimulated *in vitro* with core, E1 or E2-derived peptides and stained for surface and intracellular markers (Chapter 2 section 2.2.6.4).

The splenocytes were analysed using forward/side scatter and gated on the lymphocyte population. Single events within the lymphocytes gate were then gated based on their FSC-A and FSC-H (forward-scatter-height) linear relationship. Single events were gated on the CD44 marker to identify activated memory lymphocytes and then on CD4 and CD8 surface markers (Fig. 3.13A). The frequencies of CD4+ or CD8+ T cells secreting IL2, IFNγ or TNFα, Th1-associated cytokines previously identified as important in control of HCV infection, were then assessed. The frequency of IL4 was also assessed as a marker of a Th2-like cellular immune response (Fig 3.13B).

When stimulated with core peptides, the frequency of cytokine-producing T cells was highest in p-CE1E2-PRF vaccinated mice. The frequency of single cytokine-producing T cells peaked at 1.8% for INFγ producing CD4+ T cells (Fig. 3.14A) and 2.2% for CD8+ T cells (Fig. 3.14E), while the frequency of double cytokine-producing T cells peaked at 0.12% for TNFα/IFN-γ producing CD4+ T cells (Fig. 3.14B) and 0.065% for TNFα/IFN-γ producing CD8+ T cells (Fig. 3.14F). The frequency of triple-cytokine producing T cells peaked at 0.07% for IFN-γ/IL2/IL4 producing CD4+ T cells (Fig. 3.14C) and 0.013% for CD8+ T cells (Fig. 3.14G). The frequency of TNFα/IFN-γ/IL2/IL4 producing T cells reached 0.003% for CD4+ T cells (Fig. 3.14D) and 0.002% for CD8+ T cells (Fig. 3.14H) following stimulation with core peptides. However, none of these populations reached significance (Fig. 3.14A-H).

Following stimulation with E1 peptides, the frequency of cytokine-producing T cells was also highest in p-CE1E2-PRF-vaccinated mice. The frequency of single cytokine-producing T cells reached 0.4% for INFγ producing CD4+ T cells (Fig. 3.15A) and 0.55% for CD8+ T cells (Fig. 3.15E). The frequency of double cytokine-producing T cells peaked at 0.05% for TNFα/IFN-γ producing CD4+ T cells (Fig. 3.15B) and 0.04% for TNFα/IFN-γ producing CD8+ T cells (Fig. 3.15F), while the frequency of triple-cytokine producing T cells peaked at 0.022% for IFN-γ/IL2/IL4 producing CD4+ T cells (Fig. 3.15C) and 0.015% for CD8+ T cells (Fig. 3.15G). The frequency of TNFα/IFN-γ/IL2/IL4 producing T cells reached 0.009% for CD4+ T cells (Fig. 3.15D) and 0.011% for CD8+ T cells (Fig. 3.15H). However, none of these results was significant compared to p-CE1E2-PRF(DA) (Fig. 3.15A-H).

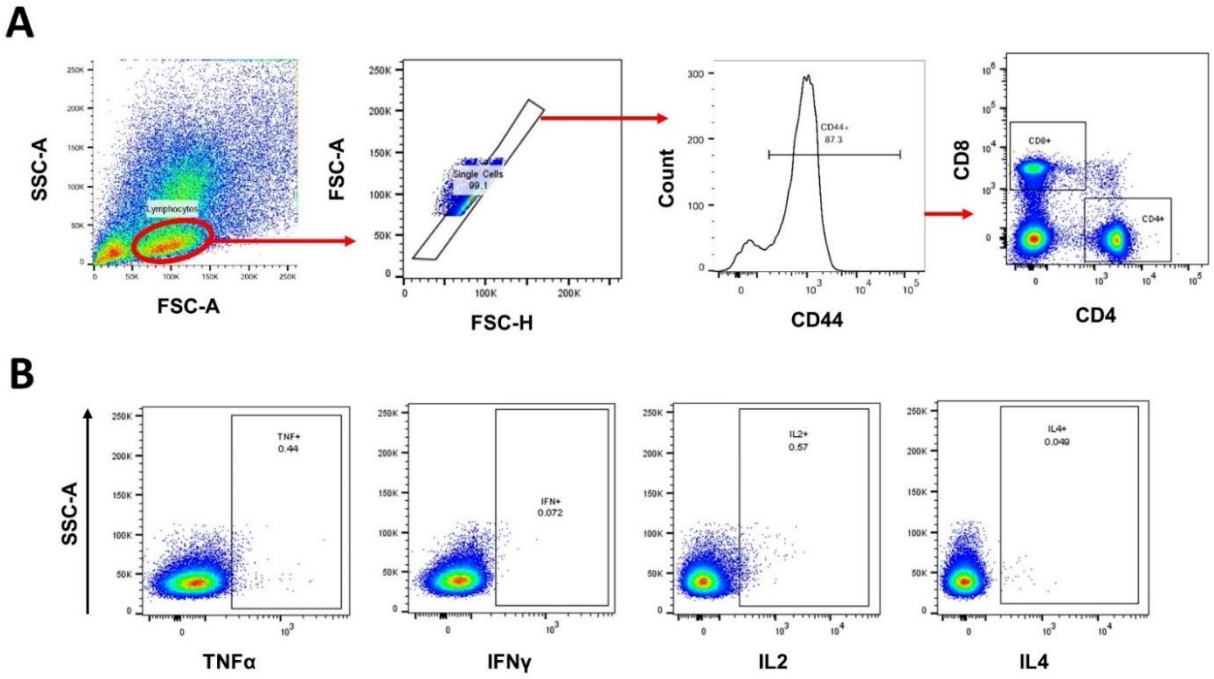


Figure 3.13. The frequency of T cell subsets in mice vaccinated with DNA vaccine, as determined by flow cytometry. Mice were vaccinated with pVax, p-CE1E2-PRF or p-CE1E2-PRF(DA) seven times at two weekly intervals with 50 µg doses and the spleens were collected 14 days later. Splenocytes were re-stimulated with core, E1, E2 pool 1 or E2 pool 2 peptides. A) The population gating for surface staining by flow cytometry. The splenocytes were initially gated on the lymphocyte gate based on the forward/side scatter and CD44+ lymphocytes, and then either on CD8+ or CD4+. B) The frequencies of IL2+, IL4+, IFNγ+ or TNFα+ T cells were determined by flow cytometry. The results were derived from 4-7 individual mice per group.

Similar results were obtained following stimulation with E2 pool 1 peptides. The frequency of single, double, triple and quadruple cytokine-producing CD4+ T cells (Fig. 3.16 A-D) and CD8+ T cells (Fig. 3.16 E-H) was slightly higher in p-CE1E2-PRF-vaccinated mice. However, these results were not significant.

Following stimulation with E2 pool 2 peptides there was a trend towards increased frequency of cytokine-producing T cells in p-CE1E2-PRF-vaccinated mice compared to those vaccinated with p-CE1E2-PRF(DA) (Fig. 3.17). The frequency of single cytokine-producing T cells peaked at 0.48% for TNFα producing CD4+ T cells (Fig. 3.17A) and 0.5% for CD8+ T cells (Fig. 3.17E). The frequency of double cytokine-producing T cells reached 0.05% for TNFα/IFN-γ producing CD4+ T cells (Fig. 3.17B) and 0.03% for TNFα/IFN-γ producing CD8+ T cells (Fig. 3.17F). The frequency of triple-cytokine producing T cells peaked at 0.02% for IFN-γ/IL2/IL4 producing CD4+ T cells (Fig. 3.17C) and 0.018% for CD8+ T cells (Fig. 3.17G). The frequency of TNFα/IFN-γ/IL2/IL4 quadruple producing T cells reached 0.004% for CD4+ cells (Fig. 3.17D) and 0.006% for CD8+ cells (Fig. 3.17H) following stimulation with core peptides. However, these results were also non-significant (Fig. 3.17A-H).

In summary, significant IFNγ release was observed in mice vaccinated with p-CE1E2-PRF following stimulation with E2 peptide pool 2. Although the highest cytokine levels were generally observed in p-CE1E2-PRF-vaccinated mice, compared to other vaccinated groups, there was no significant difference in the frequency of HCV-specific CD8 and CD4 T cells secreting IL2, IL4, or TNFα after stimulation with core, E1 or E2. Despite these disappointing results, a trend for increased cytokine levels in p-CE1E2-PRF vaccinated mice was observed.

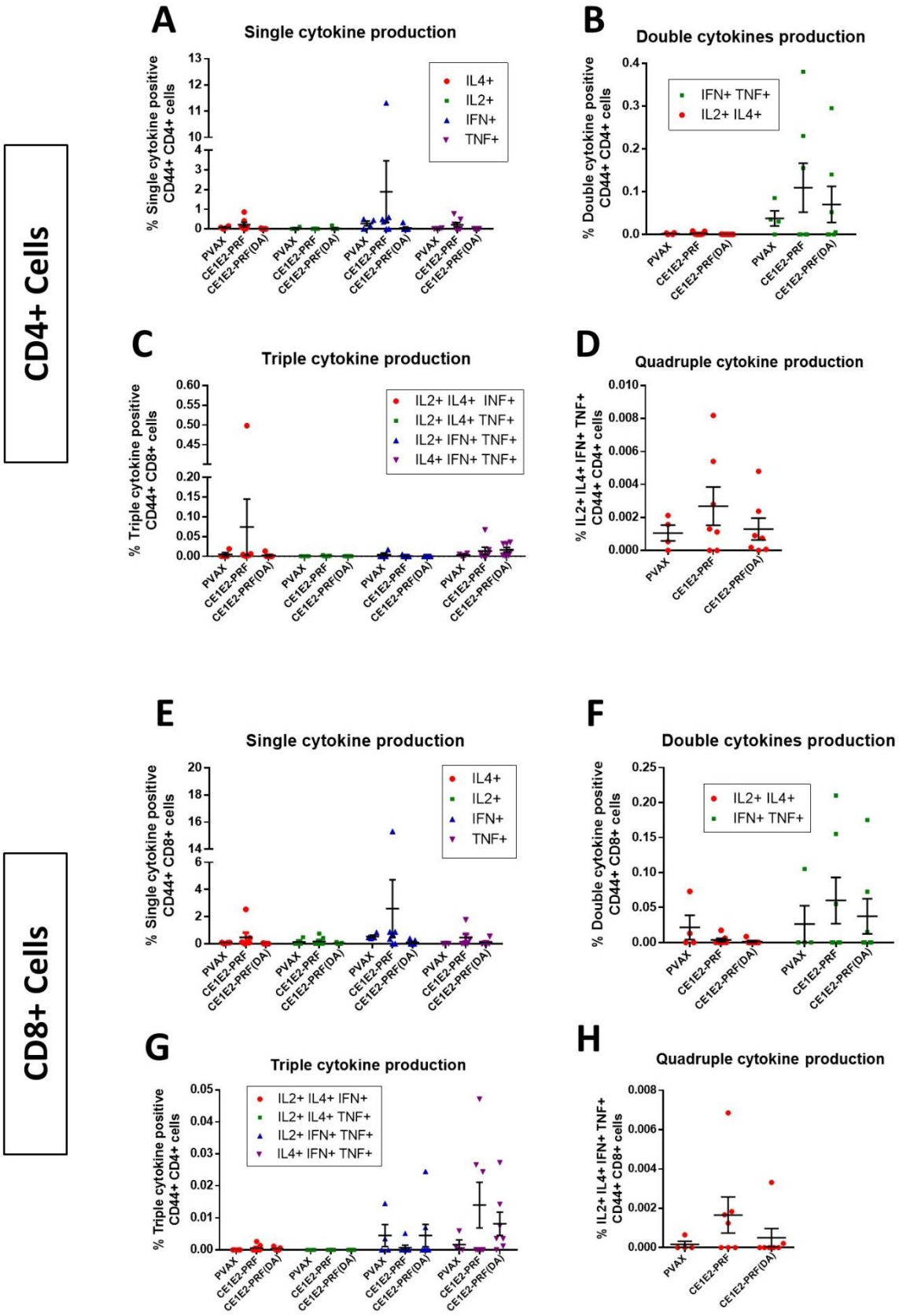


Figure 3.14. The frequency of cytokine producing T cells in mice vaccinated with DNA vaccine following stimulation with HCV-core peptides, determined by flow cytometry. 14 days post vaccination, splenocytes were re-stimulated with core peptides. The frequency of (A&E) single, (B&F) double, (C&G) triple, (D&H) quadruple producing T cells. The results are graphed from between 4-7 individual mice per group as mean ± SEM in individual mice.

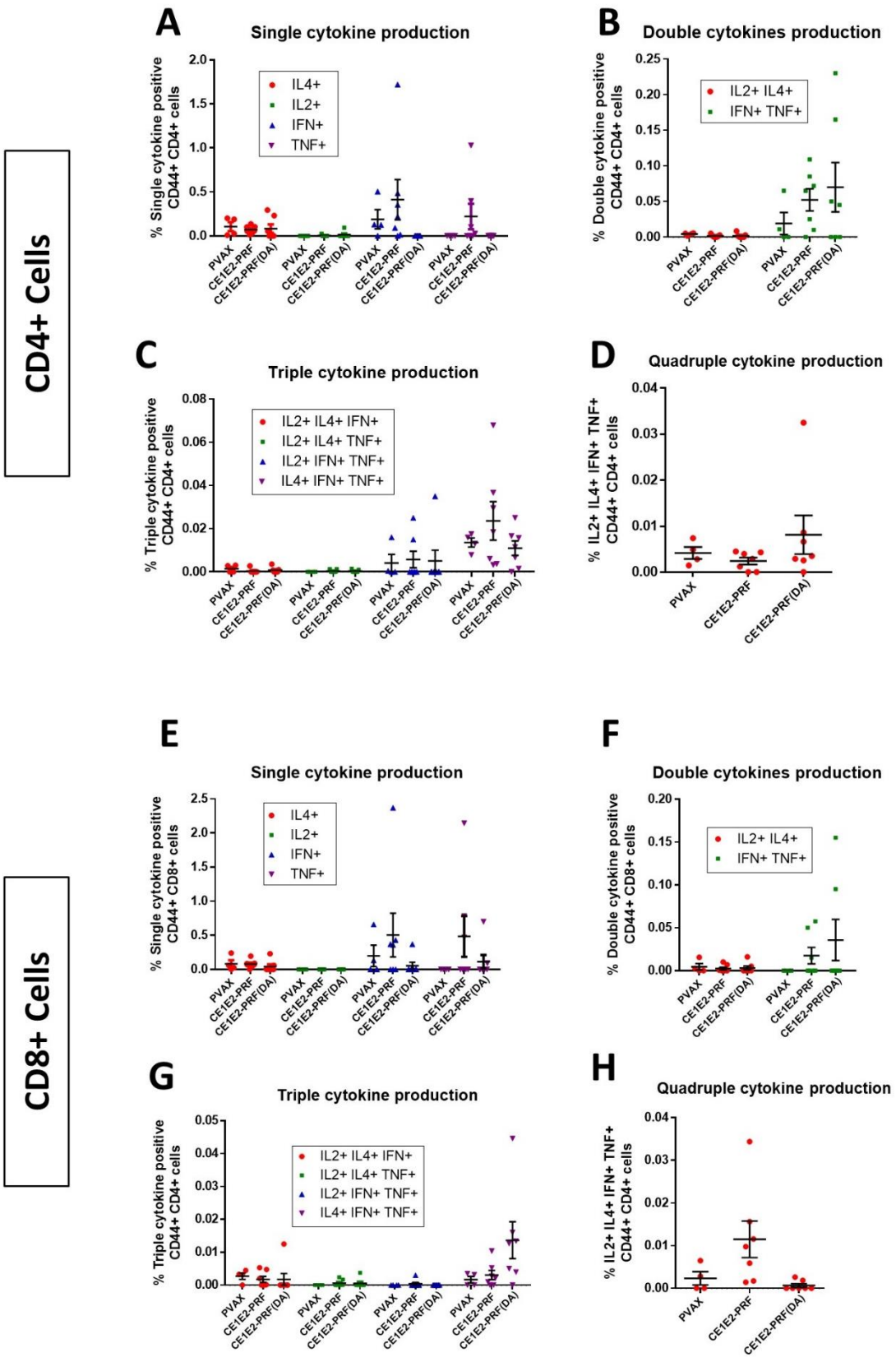


Figure 3.15. The frequency of cytokine producing T cells in mice vaccinated with DNA vaccine following stimulation with HCV-E1 peptides, determined by flow cytometry. 14 days post vaccination, splenocytes were re-stimulated with E1 peptides. The frequency of (A&E) single, (B&F) double, (C&G) triple, (D&H) quadruple producing CD4+ and CD8+ T cells. The results are graphed from between 4-7 individual mice per group as mean ± SEM in individual mice.

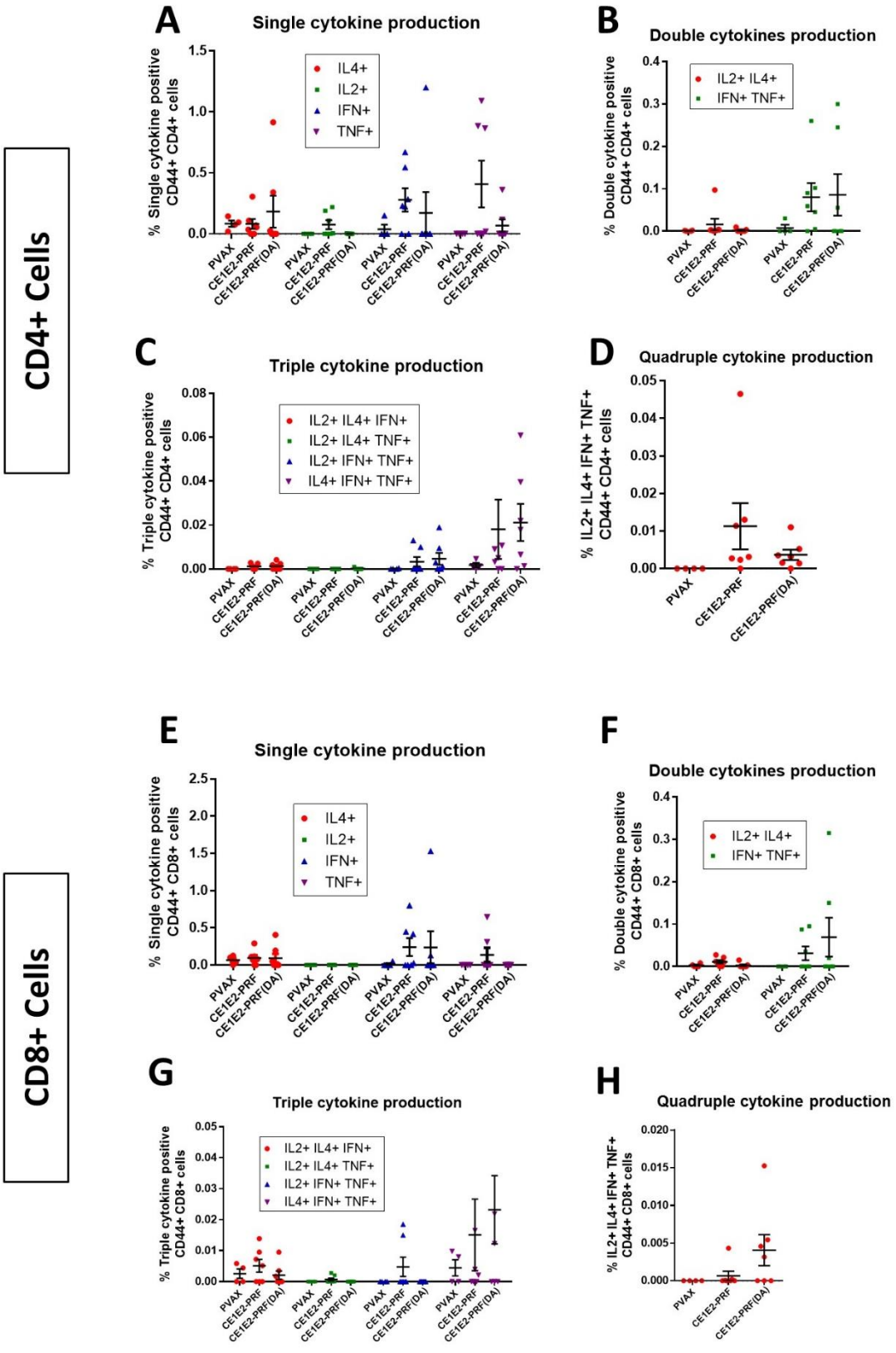


Figure 3.16. The frequency of cytokine producing T cells in mice vaccinated with DNA vaccine following stimulation with HCV-E2 pool 1 peptides, determined by flow cytometry. 14 days post vaccination, splenocytes were re-stimulated with E2 pool 1 peptides. The frequency of (A&E) single, (B&F) double, (C&G) triple, (D&H) quadruple producing CD4+ and CD8+ T cells. The results are graphed from between 4-7 individual mice per group as mean ± SEM in individual mice.

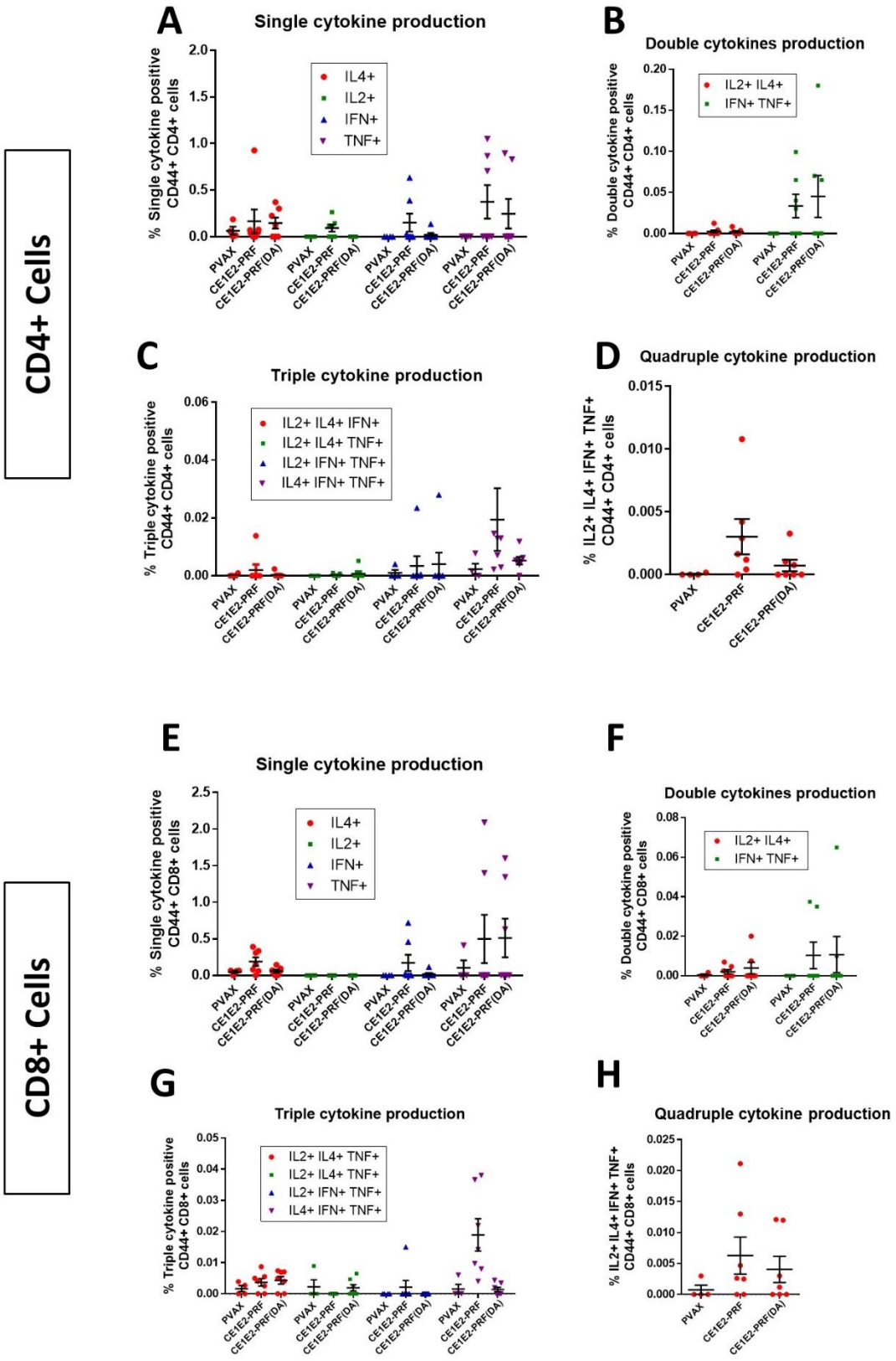


Figure 3.17. The frequency of cytokine producing T cells in mice vaccinated with DNA vaccine following stimulation with HCV-E2 pool 2 peptides, determined by flow cytometry.

14 days post vaccination, splenocytes were re-stimulated with E2 pool 2 peptides. The frequency of (A&E) single, (B&F) double, (C&G) triple, (D&H) quadruple producing CD4+ and CD8+ T cells. The results are graphed from between 4-7 individual mice per group as mean ± SEM in individual mice.

3.4 Discussion

3.4.1 Generation of DNA vaccine constructs

The use of a DNA vaccine is an attractive approach to generate antigen-specific immunity to various pathogens due to its stability and simplicity of delivery. DNA vaccines also have the ability to induce humoral and cell-mediated immune responses. Currently, there is no approved DNA vaccine for use in humans mainly due to the lack of potent immunogenicity [266]. HCV-VLPs pseudotyped with HCV structural proteins have been shown to induce strong neutralising and cell mediated immune responses in macaques [146]. Previous studies conducted in our laboratory have demonstrated that DNA vaccines adjuvanted with a cytolytic protein, perforin, have resulted in increased immune responses to the antigen [414, 415, 418, 425]. Therefore, the current chapter aimed to combine the immunogenicity of VLPs and the ease of production of plasmid DNA, by designing DNA vaccines expressing VLPs consisting of HCV-core, E1 and E2 proteins. The vaccine also encodes PRF to cause necrotic cell death and ensure the release of the VLPs from vaccine targeted cells simultaneously with DAMPs, which act as natural adjuvants to mimic the highly immunogenic effect of lytic viral infections [414, 415, 418, 425].

This chapter showed that DNA vaccines encoding Core/E1/E2 expressed from the CMV promoter and PRF or PRF(DA) expressed from the weaker SV40 promoter were successfully generated. This approach is necessary to ensure intracellular accumulation of HCV-VLPs before the threshold of PRF expression required to induce cell lysis (allowing VLP release) is reached.

3.4.2 HCV protein expression from the DNA vaccine constructs

Vaccine-derived protein expression was detected using HEK293T cells after transfection with plasmid DNA. Protein expression was then analysed with anti-core, anti-E1 or anti-E2 antibodies and bands of approximate molecular weight of ~20kDa, ~35kDa and ~66kDa were detected, corresponding to core, E1 and E2 respectively. These results correlated with the previously reported molecular weights of core, E1 and E2 [146, 420, 433-438]. The expression of these HCV-structural proteins was further confirmed by immunofluorescence analysis using core, E1 and E2 antibodies, which were shown to be confined to the cytoplasm and plasma membrane consistent with previous observations [437]. Therefore, the vaccine constructs generated in this chapter successfully expressed the HCV structural proteins

3.4.3 Perforin expression and cytolytic activity

Perforin is a cytolytic protein found in the granules of CTLs destroy target cells mainly through a perforin-dependent mechanism [439]. Transfection with the DNA vaccine resulted in successful expression of HCV-structural proteins and perforin. The results also showed that

perforin and HCV-proteins were found to be co-expressed in the same cells (Fig. 3.6, overlay). The cytolytic activity of PRF was assessed using the LDH release assay which demonstrated that cells transfected with constructs encoding PRF released significantly higher levels of LDH compared with cells transfected with the mutated PRF(DA) which has lost its cytolytic activity [440]. Additionally, cells treated with the pro-apoptotic doxorubicin released significantly low LDH levels consistent with the finding that PRF-induced cell death is likely to be necrotic rather than apoptotic [418]. Overall these results show that perforin induced cell lysis in target cells after expression, and in principle this should result in the release of VLPs that assembled intracellularly. The results also demonstrated that the mutated PRF(DA) represents an appropriate control for PRF-induced cell lysis and thus should serve as a control for the release of VLPs.

3.4.4 HCV-VLPs formation

VLPs comprised of HCV-structural proteins, are self-assembled, non-replicating, genome-free particles similar in size and shape to the intact virions and are useful tools for immunological studies. Before attempting to confirm VLP formation *in vitro*, the interaction of HCV structural proteins expressed in HEK293T cells was investigated by means of immunoprecipitation and immunoblotting.

Co-immunoprecipitation of HCV structural proteins would indicate an association of the structural proteins and potential VLP assembly. The results revealed an association between core and E2 that was detected by immunoprecipitation with anti-E2 but not anti-core antibodies. Thus, as core was precipitated with anti-E2 while E2 was not precipitated with anti-core, this suggest the formation of a particle in which the core protein was protected from direct interaction with anti-core antibodies, indicative of VLP formation. Based on these results, it can be concluded that the co-immunoprecipitation of core and E2 proteins was the result of an association of the structural proteins. These results correlate with findings of previous studies [433-436] and provide supportive data suggesting the assembly of expressed HCV structural proteins into VLPs. Moreover, the core-E2 protein association was also strongly detected in the supernatant of p-CE1E2-PRF cells suggesting the release of formed particles following PRF-induced cell lysis (Fig. 3.8C &D). Future experiment however should be conducted to further directly confirm the formation and release of these VLP using electron microscopy.

3.4.5 Immune responses

DNA vaccines are able to induce both humoral and cell mediated immune responses. However, no DNA vaccines have been licensed for use in humans and this has been attributed largely to the lack of immunogenicity. Previously, the Gowans laboratory has shown that the addition of

perforin as a genetically encoded adjuvant within the DNA vaccine has the ability to increase the immune response to HCV-NS3 [414]. The hypothesis underlying the work described in this chapter was that PRF-induced necrotic cell death will not only enhance the HCV-specific-immune responses but will also result in release of the VLPs. Mice were immunised with p-CE1E2-PRF or p-CE1E2-PRF(DA) and the HCV-specific antibody and cell mediated responses were assessed.

3.4.5.1 HCV envelope-specific antibody titres

The titres of E1E2-specific antibodies were measured in mice vaccinated with DNA encoding CE1E2 plus PRF or PRF(DA). Up to 4 boosters were required to maximally induce the anti-E1E2 response which then began to plateau (Fig. 3.11A). Vaccination with p-CE1E2-PRF resulted in significantly higher E1E2-specific antibody titers compared to p-CE1E2-PRF(DA) vaccinated mice. Due to the low levels of E1E2-specific antibodies observed, HCV-positive patient sera, as well anti-E1 and anti-E2 commercial antibodies were used as a positive control to confirm that the E1/E2 protein-coated ELISA plates were correctly formatted.

As previously noted, it was important to compare the E1E2-antibody titers with other vaccine strategies. Similar to the T cell responses, the E1 E2-antibody responses reported in this chapter were lower (mean E1E2-antibody titer of 1/35.8) compared to those reported in other studies [146, 420, 437, 438]. For example, a study using modified HCV VLPs in combination with recombinant adenovirus encoding HCV structural proteins (rAd5-CE1E2 – genotype 3a) as final booster, induced an anti-HCV titer of approximately 1/4000 [420], and a study using HCV VLPs (genotype 1a) also induced significant anti-HCV VLPs and anti-E2 responses (titers of approximately 1/3800) following vaccination in mice [421]. Furthermore, these antibodies were found to be neutralizing. This indicates that the p-CE1E2-PRF DNA vaccine is not highly effective when delivered by the ID route compared to other vaccine strategies currently under development.

Overall, these results showed that multiple injections of p-CE1E2-PRF showed low efficacy in inducing HCV-specific cell mediated and antibody responses. For this reason, and because the p-CE1E2-PRF induced E1E2-specific antibodies were unlikely to neutralize HCV particles, subsequent virus neutralisation experiments were not performed. It would however be interesting to investigate the effect of the p-CE1E2-PRF vaccine constructs in a prime-boost strategy, for example using a rAd5-CE1E2-based HCV vaccine or purified HCV VLPs [420], as this may further enhance HCV-specific immune response to levels similar to those reported in the literature.

3.4.5.2. Cell mediated immunity

IFNγ secretion is a marker of a Th1 type antigen-specific cellular immune response that plays a key role in fighting virus infection. Vaccination with p-CE1E2-PRF increased the HCV-specific cell-mediated immune response relative to p-CE1E2-PRF(DA), as measured by IFNγ ELISPOT and intracellular cytokine staining. Specifically, p-CE1E2-PRF vaccination enhanced IFNγ ELISPOT responses to E2 pool 2 peptides (Fig. 3.12D). Vaccination with p-CE1E2-PRF also increased the frequencies of single, double, triple or quadruple cytokines producing CD8+ and CD4+ T cells secreting IL-2, IL-4, IFNγ or TNFα simultaneously (Fig. 3.14 – 3.17), representing an increase, although not significant, in multi-functional T cells which have been shown to be associated with protective immune responses during chronic HCV infection [432].

This chapter shows that encoding PRF in the vaccine construct resulted in an increase in HCV-specific immune responses, compared to PRF(DA), after ID DNA vaccination. However, it is important to consider these results in the context of other studies, and to determine how they compare to other novel vaccine strategies currently under development. The core, E1 and E2-specific ELISPOT results described in this chapter were of a low frequency (up to an average of 58 SFU/10^6 cells for E1 peptides) and did not compare well to those reported in other studies using CE1E2 antigen from genotype 1a [441]; 1b [420, 441, 442] or 3a [420]. This low response could be attributed to various factors, including an insufficient uptake of the DNA vaccine *in vivo* by cells in the intradermal environment or an insufficient or partial release of the VLPs *in vivo* following PRF-induced cell death, leading to insufficient priming of the HCV-specific response.

The multiple-cytokine responses measured by flow cytometry were difficult to compare with other publications because most studies of HCV vaccines encoding core, E1 and E2 failed to assess the HCV-specific cytokine profile of T cells, at least to the degree it was assessed in this study. However, recent studies have reported total HCV-specific IFNγ-positive cell frequencies of 0.3-8% of total CD8+ T cells [420, 441, 442] and in the experiments described in this chapter, the IFNγ-positive frequencies were 0.2-2.5%. Data on the frequency of TNFα, IL-2, IL-4, double, triple or quadruple cytokine-positive T cell following immunisation with a vaccine encoding core, E1 and E2 proteins were not available in the literature.

3.5 Conclusion

The results in this chapter describe the synthesis of bicistronic DNA vaccine constructs encoding HCV structural proteins and the cytolytic gene perforin (controlled by the SV40 promoter) which has the ability to i) induce necrotic cell death, ii) result in release of the

assembled VLPs into the extracellular environment and iii) serve as an adjuvant. p-CE1E2-PRF-vaccinated mice generated higher responses than those vaccinated with p-CE1E2-PRF(DA), confirming an adjuvant effect by PRF. However, these responses were weak compared to those reported in the literature and are unlikely to be effective. This project originally intended to induce neutralising antibodies using p-CE1E2-PRF. However, given the low antibody titers observed with these DNA vaccine constructs, it was decided to use novel DNA vaccine formulations encoding E1E2 heptamers described in the following chapters.

Chapter 4. Generating and assessing the immunogenicity of DNA vaccine constructs encoding secreted HCV-E1 and E2 coupled to IMX313

4.1 Introduction

The HCV virion is composed of a nucleocapsid surrounded by a cell-derived membranous envelope that contains the glycoproteins E1 and E2. The envelope proteins are the target of neutralising antibodies (NAbs) because they are exposed on the surface of the virion and are involved in essential initial steps in the replication cycle of the virus including viral attachment/entry or membrane fusion. Therefore, E1 and E2 represent the most immunologically significant HCV antigens and have been the main focus of various HCV vaccine development studies [146, 420, 443-445]. Antibodies that can prevent E1/E2 from binding to HCV receptor/entry factors are thought to be neutralising. HCV glycoproteins E1 and E2 are type I membrane proteins with a C-terminal TMD anchored in the virion phospholipid envelope [446]. In their functional form, E1 and E2 form a non-covalent heterodimer, and their TMDs are essential for heterodimerisation [447, 448]. Immunolocalization studies and glycan analyses have shown that the HCV glycoproteins are located in the endoplasmic reticulum (ER) of infected cells [449, 450].

Deletion of the transmembrane (TM) domains of E1 and E2 resulted in the secretion of these truncated forms of HCV glycoproteins into the extracellular medium [451-454] and a decrease in the cellular immune response to the antigen [455]. Others have shown that in its secreted form, the E2 antigen leads to an increased humoral response in mice [456]. Conversely, it is difficult to elicit anti-E1 responses which require the dissociation of E1E2 expression, suggesting that E2 is immunodominant or that immunogenic E1 epitopes are masked in the presence of E2 [146]. This is further supported by a recent study using a vaccine candidate based on chimeric HBV-HCV envelope particles, in which specific anti-E1 and anti-E2 antibody responses were profoundly impaired in animals immunized with vaccine particles harbouring an E1E2 heterodimer with respect to animals immunized with particles harbouring E1 and E2 separately [147]. Additionally, the anti-E1 and anti-E2 antibodies were reported to have additive neutralizing properties that increased the cross-neutralization of heterologous strains of various HCV genotypes, highlighting the importance of including E1 and E2 as separate immunogens to induce a dual anti-E1 and anti-E2 response [147].

Oligomerisation is employed by many natural proteins to increase protein valency, binding affinity and structural stability [457]. The complement inhibitor C4b-binding protein (C4 bp)

is a potent circulating soluble inhibitor of the classical and lectin pathways of complement by controlling the relative levels of c3 convertase [458]. This protein is produced by hepatocytes and is mainly found in the plasma of many organisms including humans [459]. The major form of C4 bp in plasma consists of seven identical α-chains and one β-chain linked at the C-terminus [458]. Both types of subunits are composed of complement control protein (CCP) domains, eight such domains make up one α-chain [460]. In addition, the 57 amino acids of the C-terminus of the α-chain contains an amphiphatic α-helix region that is required for polymerisation [459]. These protein oligomers are stabilised by the cysteine residues located within the α-chains [458]. Studies assessing the efficacy of vaccines containing protein antigens fused to the C4 bp protein as an adjuvant showed promising results [461-463]. The most effective domain was found to be a hybrid C4 bp, known as IMX313, which was genetically modified to contain a chicken C4b-p motif and < 20% similarity to human and murine C4 bp [461]. Fusion of protein antigens to the IMX313 oligomerisation domain, was shown to spontaneously form soluble heptameric structures following expression, resulting in improved antibody response compared to the same dose of monomeric antigen [461-463]. Furthermore, mice vaccinated with the malaria vaccine candidate MSP119 fused to IMX313 were protected against challenge with a lethal dose of *Plamodium yoelii* parasites [461]. Other studies have demonstrated that immunisation of mice with the *Mycobacterium tuberculosis* antigen 85A fused to IMX313 in both DNA and viral vector vaccines resulted in consistently increased CD4+ and CD8+ T cell responses in mice and improved magnitude of the immune response in mice and non-human primates without a reduction in quality [462]. While oligomerisation may be an important element, the mechanism by which the C4b-p enhances immunogenicity is still not well understood [461, 464]. Nevertheless, some studies have suggested that the C4 bp protein acts as a cofactor to the serine protease factor 1, required for the proteolytic activation of C4b, preventing the formation and reconstitution of the classical C3-convertase (C4bC2a) [460, 465]. A recent phase I clinical trial of a viral vector encoding 85A-IMX313 in healthy BCG (Bacillus Calmette-Guerin)-previously vaccinated UK adults revealed that the vaccine was well tolerated and immunogenic [426] (clinicaltrials.gov ref. NCT01879163). The Gowans laboratory has developed a potential Tat-based HIV DNA vaccine, encoding HIV-tat fused to IMX313, capable of eliciting high titer anti-Tat NAbs and CMI [466].

4.2 Aims

This aims of this chapter are to:

1. Construct DNA vaccines encoding secreted E1 and E2 fused to IMX313
2. Confirm E1 and E2 expression and oligomerisation from the different constructs *in vitro.*

3. Assess the immunogenicity of E1 and E2 proteins as separate immunogens or as a single E1E2 polyprotein when fused to IMX313.

4.3 Results

4.3.1 Plasmid construction

To generate the plasmid encoding secreted forms of E1 and E2 fused to the IMX313, the TMDs of E1 and E2 were removed in a series of overlapping PCRs (Fig. 4.1 – Chapter 2 -section 2.2.2.3) using primers designed to generate individual fragments with overlapping overhangs which were used to fuse the fragments together (Fig. 4.2). Similarly, the signal peptide sequence of the tissue plasminogen activator (tPA) was introduced as a leader sequence to drive E1/E2 proteins into the cellular secretion pathway. Fragments encoding tPA, E1, E2 and IMX313 fusion proteins were cloned into pVax. The expression of the secreted E1 and E2 proteins and the oligomerisation of IMX313 was examined *in vitro* prior to immunogenicity studies in mice.

The plasmids p-tPA-HIVTat-IMX313 [466] and p-CE1E2-PRF were used as templates to generate six PCR fragments with overlapping overhangs. p-tPA-HIVTat-IMX313 was used to amplify tPA and IMX313 sequences while p-CE1E2-PRF was used to generate the truncated E1 (tE1), E2 (tE2) genes lacking the TMDs.

The different fragments with overlapping ends were subsequently used in an overlap-fusion PCR (Fig. 4.1) to generate DNA fragments encoding tPAtE1, tPAtE2, tPAtE1tE2, tPAtE1IMX313, tPAtE2IMX313 or tPAtE1tE2IMX313 containing the NheI and EcoRI restriction enzyme sites. Following digestion with NheI and EcoRI these fragments were individually inserted downstream of the CMV promoter in pVax. DH5α cells were transformed using the ligation mix and colony PCR was performed to identify clones with the desired amplicon. Positive clones were further analysed by restriction enzyme digestion. The sequences of the positive clones were confirmed by DNA sequencing. A complete catalogue of plasmid maps used in this chapter is shown in Figure 4.3.

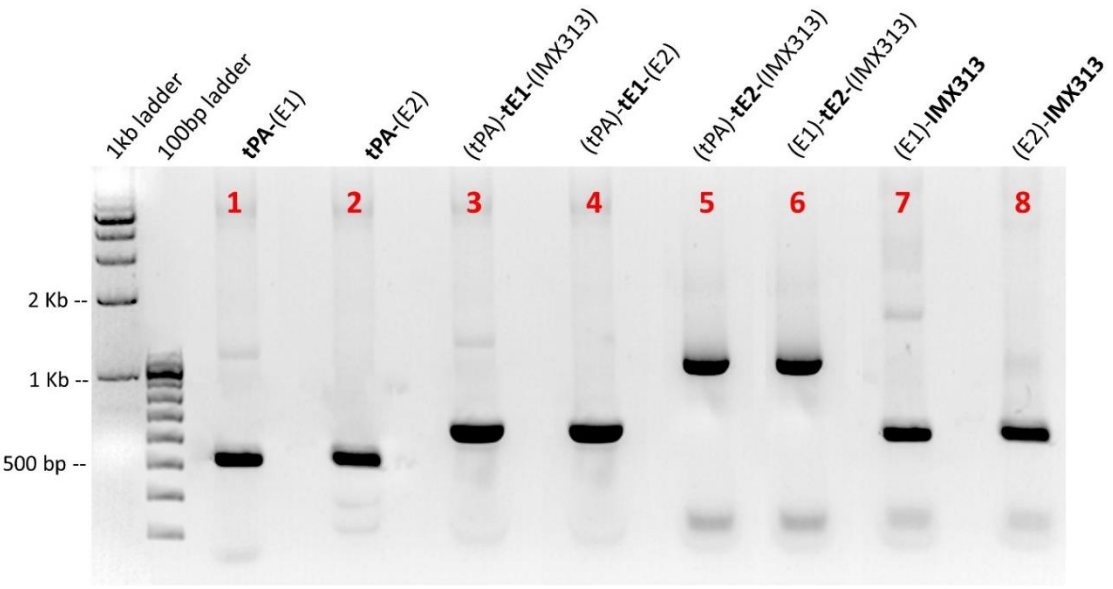


Figure 4.2. The production of chimeric tPA, truncated E1 and E2 and IMX313 genes. Gel electrophoresis analysis of the tPA, truncated HCV-E1 and E2 and IMX313 genes amplified by PCR with chimeric overhangs shown in brackets. These fragments were fused in a subsequent PCR to generate single PCR products. To generate tPAtE1IMX313, fragment 1, 3 and 7 were used. Fragment 2, 5 and 8 were used to generate tPAtE2IMX313, while fragments 1, 4, 6 and 8 were used to generate tPAtE1tE2IMX313. Fragments without the IMX313 gene were amplified using p-tPAtE1IMX313, p-tPAtE2IMX313 or p-tPAtE1tE2IMX313 as templates to generate p-tPAtE1, p-tPAtE2, p-tPAtE1tE2 plasmids.

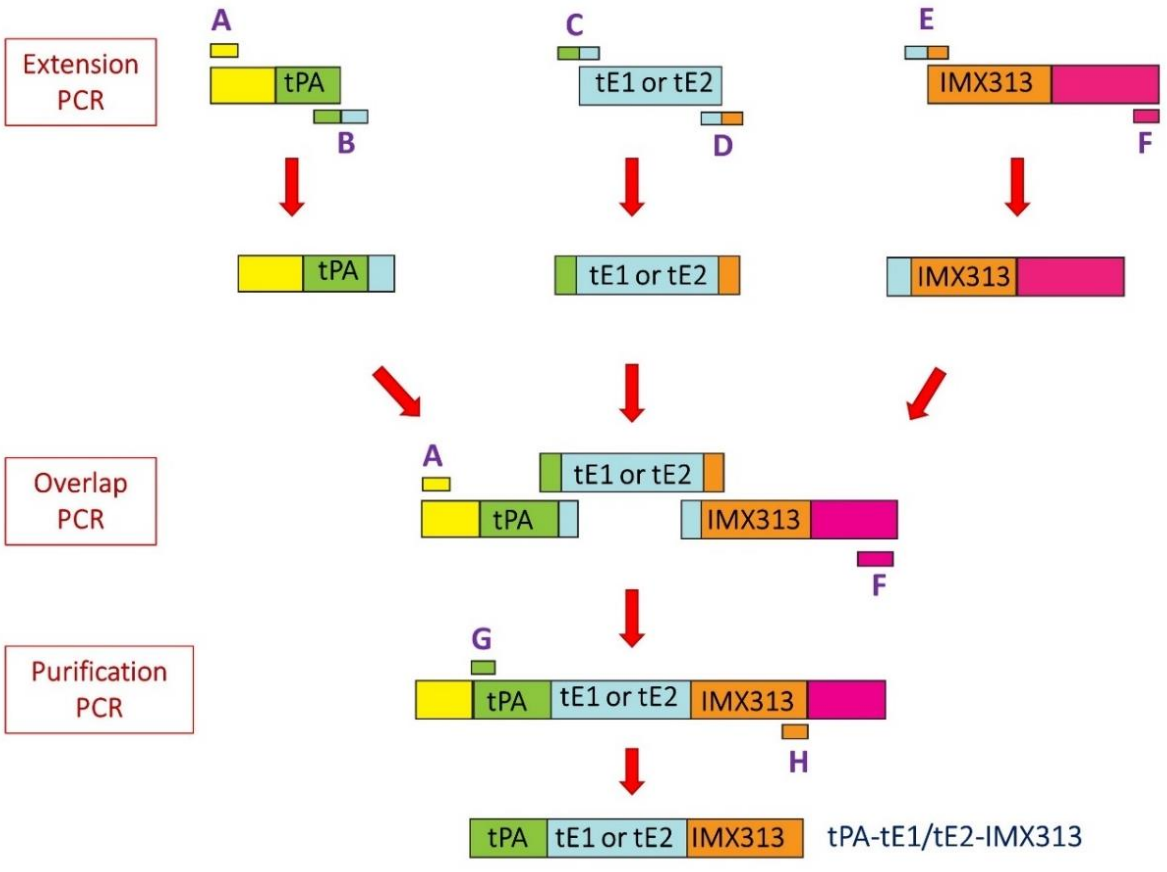


Figure 4.1. Schematic of the strategy used to generation of secreted truncated E1/E2-IMX313 genes. During an extension PCR individual fragments encoding tPA, truncated E1 and E2 and IMX313 are amplified using primers with overlap sequences to allow fusion. The amplified fragments were ligated at the overlap segments during the overlap PCR forming a single large construct. The ligated products are then amplified (purification PCR) using another set of primers containing restriction enzyme site to generate tPAtE1IMX313, tPAtE2IMX313 or tPAtE1tE2IMX313 products.

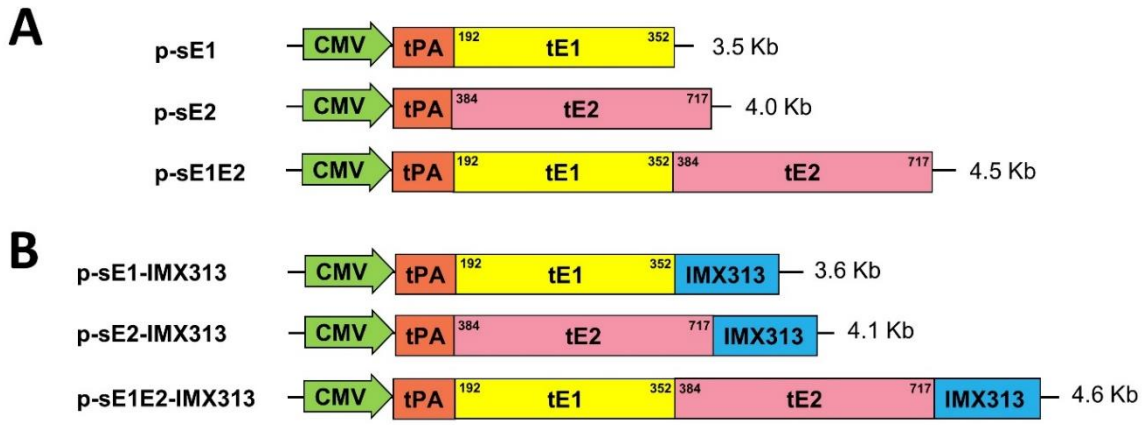


Figure 4.3. Plasmid maps of DNA constructs used in this chapter. A) DNA plasmids encoding secreted and truncated E1 and E2 B) DNA vaccine constructs produced by fusing the IMX313 multimerization domain to the 3' end of the genes encoding secreted tE1 or tE2 proteins. The numbering corresponds to the amino acid position in the HCV polyprotein. DNA plasmid sizes are shown on the right.

4.3.2 Detection of HCV envelope proteins and IMX313 oligomerisation

The expression of E1 and E2 in HEK293T cells transfected with p-tPAtE1, p-tPAtE2, p-tPAtE1tE2, p-tPAtE1IMX313, p-tPAtE2IMX313 or p-tPAtE1tE2IMX313 was initially assessed by immunofluorescence analysis using anti-HCV serum from infected patients (Fig. 4.4). Staining was observed in cells transfected with all DNA constructs, apart from the pVax (empty) control as expected. Cells transfected with the same DNA constructs were stained with normal human serum to detect non-specific background staining (Fig. 4.4 - right panels).

To confirm expression of the HCV envelope proteins and oligomerisation of E1/E2-IMX313 fusion proteins, HEK293T cells were transfected with the plasmid DNA constructs, and western blot analysis was performed on supernatant fluids from p-tPAtE1, p-tPAtE2, p-tPAtE1tE2, p-tPAtE1IMX313, p-tPAtE2IMX313 or p-tPAtE1tE2IMX313 transfected cells under reducing (Fig. 4.5A) and non-reducing conditions (Fig. 4.5B). The proteins migrated as monomers under reducing conditions with molecular weights of ~35 kDa for E1 and ~68 kDa for E2. In non-reducing western blot, high-molecular weight forms of E1 and E2 were detected with molecular

weights exceeding 250 kDa in samples which contained HCV envelope-IMX313 fusion proteins. Under non-reducing conditions, proteins from p-tPAtE1, p-tPAtE2 and p-tPAtE2tE2 transfected cells showed bands of approximately ~50 kDa for E1 and ~80 kDa for E2. Additionally, faint bands of approximately ~75 kDa for p-tPAtE1 and ~120 kDa for p-tPAtE2 and p-tPAtE2tE2 were also observed (Fig. 4.5B). No HCV-specific bands were detected in pVax-transfected cells or in the cell controls. Overall these results demonstrate that soluble versions of tE1/tE2 fused to the IMX313 domain were efficiently produced and secreted into the culture medium.

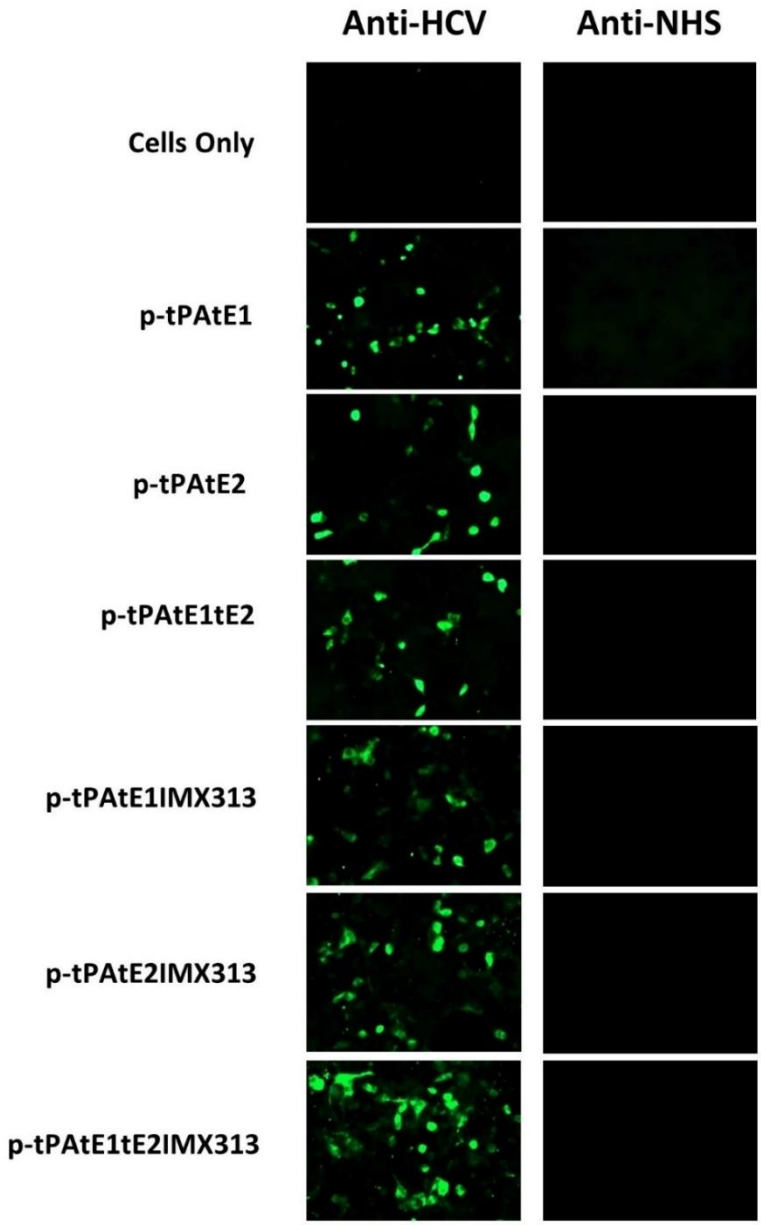


Figure 4.4. Immunofluorescence of HCV envelope proteins in HEK293T cells. Fluorescence staining of HEK293T cells transfected with p-tPAtE1, p-tPAtE2, p-tPAtE1tE2, p-tPAtE1IMX313, p-tPAtE2IMX313 and p-tPAtE1tE2IMX313. Approximately 48 hours post transfection cells were fixed/permeabilised and probed with Anti-HCV (pooled HCV genotype1 patient sera, green) and anti-NHS (normal human serum) as a control for cross reaction and unspecific background. Untransfected cells were used as control non-specific fluorescence.

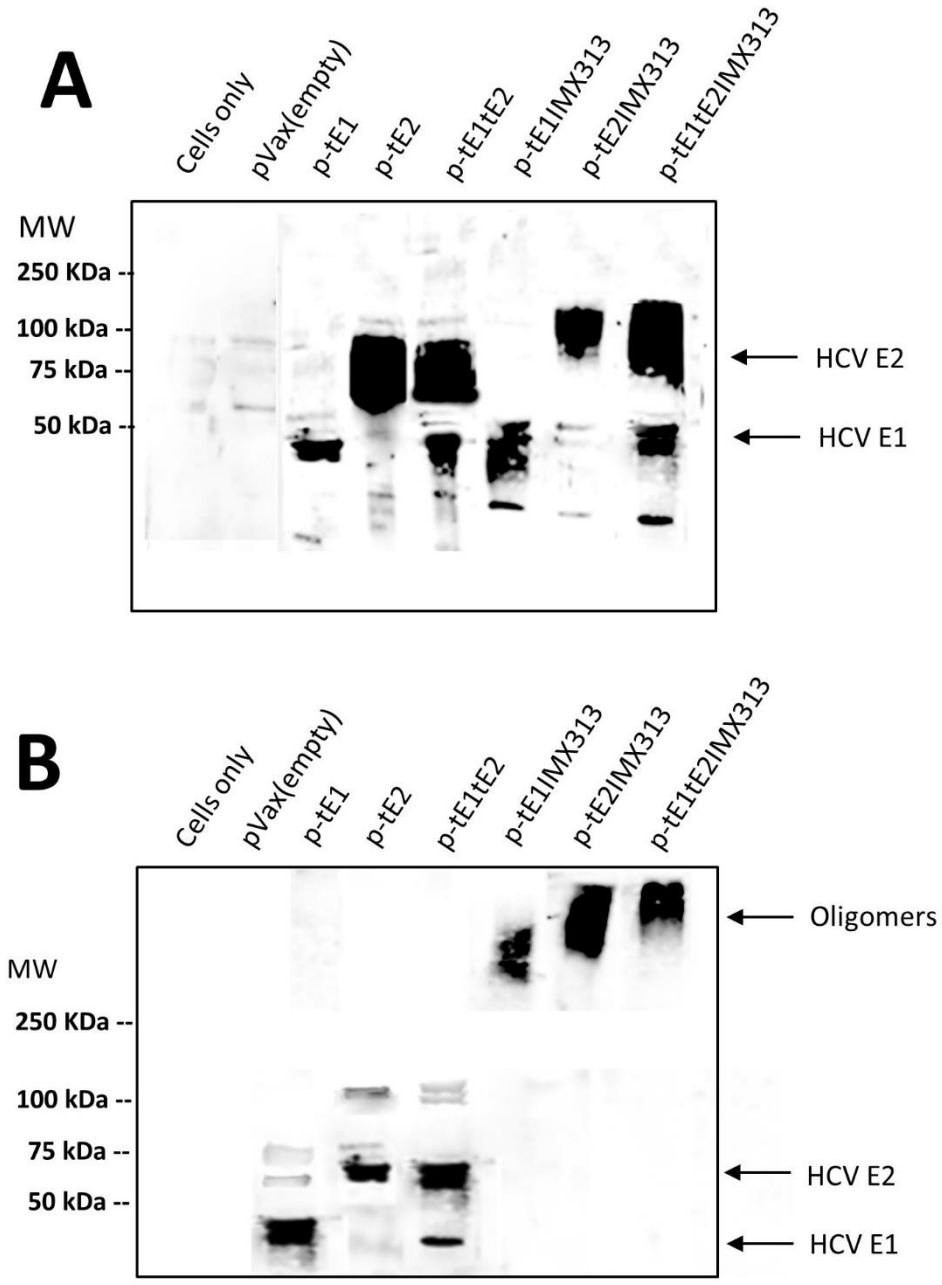


Figure 4.5. Expression of HCV envelope proteins from the DNA vaccines. HEK293T cells were transfected with p-tPAtE1, p-tPAtE2, p-tPAtE1tE2, p-tPAtE1IMX313, p-tPAtE2IMX313 or p-tPAtE1tE2IMX313. At 72 hours post transfection, cell culture media was harvested, centrifuged to remove detached cells then filtered through a 0.22 µm filter to remove remaining cell debris. Clarified supernatants were concentrated by ultrafiltration through a 70,000-molecular-weight cutoff filter (70K MWCO; Amicon). The protein samples were then subjected to SDS-PAGE and immunoblotting under (A) reduced and (B) non-reduced conditions, and stained with anti-HCV antibodies. Molecular masses (in kilodaltons) of protein molecular weight (MW) markers are indicated on the left; HCV-specific proteins are indicated on the right. The untransfected cells and pVax-transfected cells represent controls to show non-specific bands.

4.3.3 Animal vaccinations

Female mice, aged 6-8 weeks old were grouped into 7 mice per group and were vaccinated with a cocktail of p-tPAtE1 + pVax, p-tPAtE2 + pVax, p-tPAtE1 + p-tPAtE2, p-tPAtE1IMX313 + pVax, p-tPAtE2IMX313 + pVax, p-tPAtE1tE2IMX313 + pVax or p-tPAtE1IMX313 + -tPAtE2IMX313. pVax was included in the vaccine cocktail to ensure that all animals received an equimolar concentration of DNA to those vaccinated with p-tPAtE1 + p-tPAtE2 or p-tPAtE1IMX313 + p-tPAtE2IMX313. The remaining mice were administered pVax (empty) as a control (Fig. 4. 6A). Mice were vaccinated 6 times every 3 weeks via the intradermal route (Fig. 4. 6B). Each group of animals was vaccinated with a total of 20.65 picomoles per dose per animal. The vaccine dose was determined using 50µg DNA of the smallest construct, p-tPAtE1, to establish the baseline molecular mass for vaccinations as stated in chapter 2 - section 2.2.5.2. This ensured that each group received equimolar doses of each plasmid. Mice were bleed on a weekly basis and the serum was analysed for the presence of antibodies against HCV VLPs. Mice were sacrificed 21 days after the final vaccination, the spleen removed and splenocytes prepared for IFN-y ELISPOT analysis.

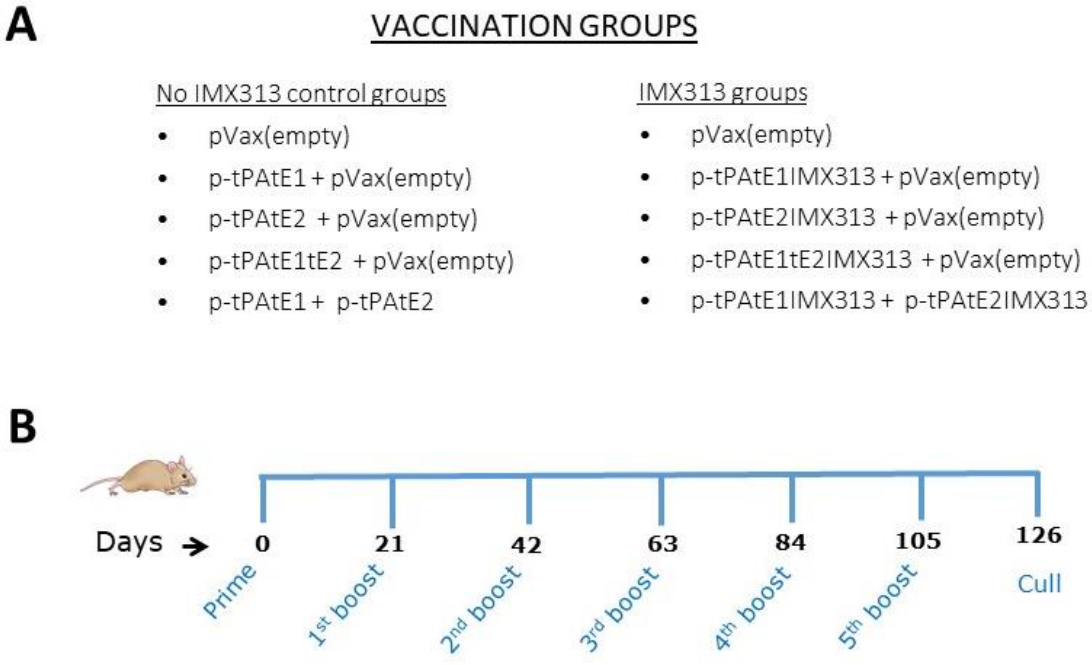


Figure 4.6. Vaccination in 6-8 weeks old female Balb/C mice. Vaccination groups (A) and immunisation schedule of animals (B). Female Balb/C mice (n= 7/group) were inoculated intradermally with 20.65 picomoles of respective plasmids at three weekly intervals. pVax was included in the vaccine cocktail to ensure that all animals were vaccinated with an equimolar concentration of DNA. Blood was collected weekly and spleens were harvested 21 days post the final vaccination.

4.3.4 Immune responses

4.3.4.1 Humoral immune responses elicited by DNA vaccines encoding secreted E1/E2-IMX313

To determine whether the DNA vaccine encoding secreted E1/E2-IMX313 fusion protein elicited antibody responses in mice, mouse sera collected after each vaccination were analysed for E1E2-specific antibodies by ELISA. The results showed that, of the constructs without the IMX313 oligomerisation domain, vaccination with p-tPAtE2 combined with pVax resulted in the highest anti-E1E2 response while the antibody levels peaked and reached a plateau following the 3rd booster immunisation (Fig 4.7A). However, titers measured 3 weeks post the last vaccination only showed an average titer of 1/5.7 while mice vaccinated with a cocktail of p-tPAE1 + ptPAtE2 showed an average titer of 1/8.5 (Fig. 4. 7B). The antibodies generated by immunisation with the DNA vaccine constructs also bound to full length E1E2 protein in an immunofluorescence analysis of HEK29T cells transfected with p-CE1E2-PRF(DA) using sera from vaccinated mice as the primary antibody (Fig. 4.7C and Fig. 4.8C).

Following vaccination with a DNA vaccine encoding secreted E1/E2-IMX313 fusion protein, animals that received a cocktail of p-tPAtE2IMX313 + pVax, p-tPAtE1IMX313 + p-tPAtE2IMX313 or p-tPAtE1tE2IMX313 + pVax resulted in the highest anti-E1E2 responses (Fig. 4. 8A). The antibody levels also peaked and reached a plateau following the 3rd booster immunisation. As shown in Figure 4.9B, antibody titers measured 3 weeks after the final vaccination revealed that immunisation with a cocktail of p-tPAtE1IMX313 + p-tPAtE2IMX313 resulted in the highest anti-E1E2 antibody response with an average titer of 1/110.7, followed by p-tPAtE2IMX313 + pVax-vaccinated mice (average titer 1/63), p-tPAtE1tE2IMX313 + pVax (average titer 1/18.4) and finally p-tPAtE1IMX313 + pVax (average titer 1/3.7) (Fig. 4. 8B). The antibodies from the cocktail DNA vaccinated mice were also shown to bind to full length E1E2 polyprotein as a heterodimer in an immunofluorescence analysis (Fig. 4. 8C). Collectively, the data presented in this chapter indicate that DNA vaccines encoding E1/E2-IMX313 were able to elicit antibody responses to the HCV envelope proteins, although the titers were comparatively low.

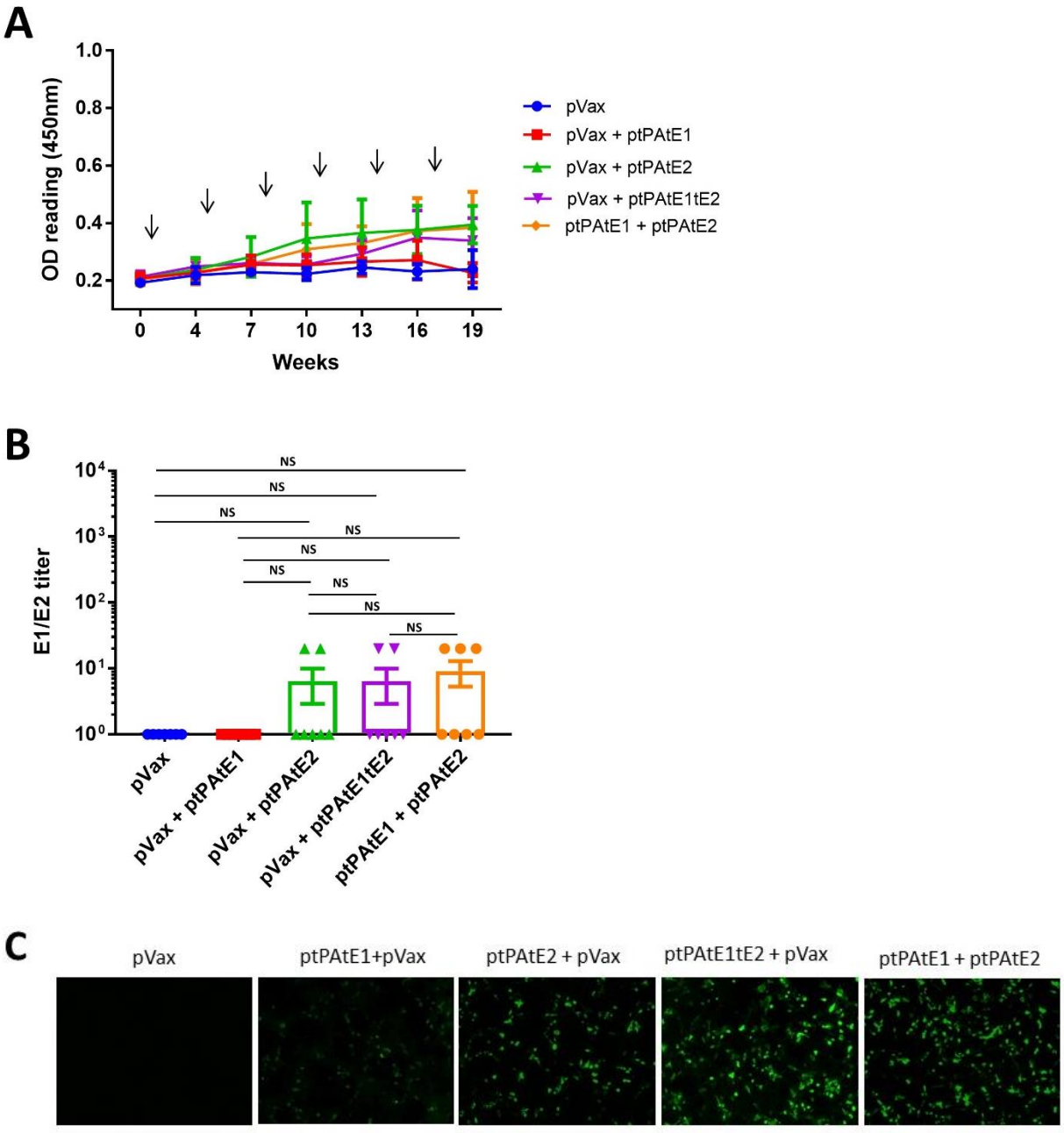


Figure 4.7. Induction of anti-E1 and anti-E2 antibodies following vaccination with p-tPAE1, p-tPAtE2 or p-tPAtE1tE2. A) Serum antibody response specific for HCV E1E2 proteins. Serum from vaccinated mice was diluted (1/50) and anti-E1E2-specific antibodies were measured by ELISA at the indicated time points. Black arrows indicated when the vaccine was administered. Values represent mean responses in each group (n = 7) ± SEM. B) Three weeks after the final immunisation, serum from individual mice was serially diluted, and endpoint binding titers were calculated. Values for individual mice are shown (n = 7) and bars represent the mean ± SEM. Mann-Whitney non-parametric t-test was performed to assess significant p-values between the vaccinated groups. C) Immunofluorescent analysis of the presence of antibodies specific for full-length E1 and E2 proteins in immune sera. HEK293T were transfected with p-CE1E2-PRF(DA) encoding full length E1E2 and pooled sera from vaccinated p-tPAE1, p-tPAtE2 or p-tPAtE1tE2 were used as the primary antibody at a 1:50 dilution.

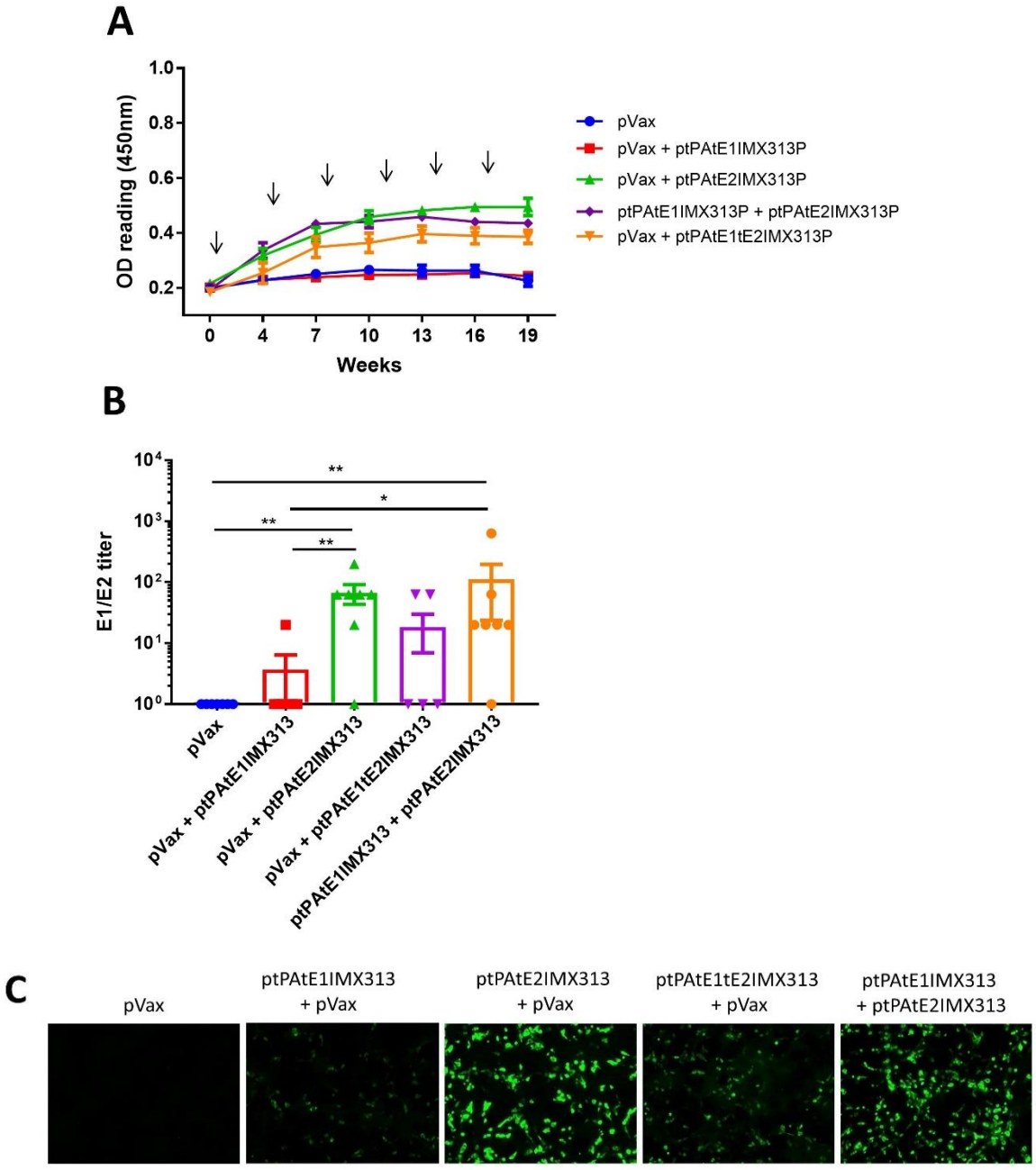


Figure 4.8. Induction of anti-E1 and anti-E2 antibodies following vaccination with p-tPAE1IMX313, p-tPAtE2IMX313 or p-tPAtE1tE2IMX313. A) Serum antibody response specific for HCV E1E2 proteins. Serum from vaccinated mice was diluted (1/50) and anti-E1E2-specific antibodies was measured by ELISA at the indicated time points. Black arrows indicated when the vaccine was administered. Values represent mean responses in each group (n = 7) ± SEM. B) Serum from individual mice (3 weeks after the final immunisation) was serially diluted, and endpoint titers for immunised mouse sera were calculated. Values for individual mice are shown (n = 7) and bars represent the mean ± SEM. Mann-Whitney non-parametric t-test was performed and significant p-values between the vaccinated groups are shown. *$p<0.05$, **$p<0.01$. C) Immunofluorescent analysis of the presence of antibodies specific for full-length E1 and E2 proteins in immune sera. HEK293T were transfected with p-CE1E2-PRF(DA) encoding full length E1E2 proteins and pooled sera from mice immunised with p-tPAE1IMX313, p-tPAtE2IMX313 or p-tPAtE1tE2IMX313 were used as the primary antibody at a 1:50 dilution.

4.3.4.2 Cells mediated immune responses elicited by DNA vaccine encoding secreted E1/E2-IMX313 in mice

Induction of cellular immune responses against the E1 or E2 proteins was assessed by IFNγ ELISPOT. Splenocytes were harvested 2 weeks after the final immunization and stimulated with HCV peptide pools representing the E1 or E2 proteins. In general, vaccination with p-tPAtE1 + pVax or p-tPAtE2 + pVax generally resulted in slightly higher E1- or E2-specific cell mediated responses compared to vaccination with p-tPAtE1 + p-tPAtE2 (Fig. 4.9). Similarly, the groups vaccinated with a combination of p-tPAtE1IMX313 + pVax or p-tPAtE2IMX313 + pVax developed higher SFUs than the groups vaccinated with ptPAtE1tE2IMX313 or the combination of ptPAtE1IMX313 + ptPAtE2IMX313 (Fig. 4.10). As expected when stimulated with E1 peptides, p-tPAtE2 and p-tPAtE2IMX313 vaccinated animals showed significantly lower IFNγ responses (Fig. 4.9A and Fig 4.10A). Similarly, p-tPAtE1 + pVax and p-tPAtE1IMX313 + pVax vaccinated mice respectively generated significantly lower IFNγ responses following stimulation with E2 peptides (Fig. 4. 9B & C and Fig 4.10B & C).

Following stimulation with E1 peptides, mice immunised with p-tPAtE1 + pVax or p-tPAtE1 + pVax showed significant numbers of IFN-γ-secreting T cells with 77.3 SFU/10^6 cells and 111.3 SFU/10^6 cells respectively. The highest overall response was seen in splenocytes from mice vaccinated with ptPAtE1IMX313 + pVax (203 SFU/10^6 cells), followed by mice that received the combination of ptPAtE1IMX313 + ptPAtE2IMX313 (115 SFU/10^6 cells) or ptPAtE1tE2IMX313 + pVax (91 SFU/10^6 cells) (Fig. 4.10A). The pVax(empty), ptPAtE2 + pVax or ptPAtE2IMX313 + pVax groups all showed significantly lower IFNγ responses as expected.

After the stimulation of the cells with the E2 pool 1 peptides, significant responses were detected in p-tPAtE2 + pVax (67 SFU/10^6 cells) and p-tPAtE1 + p-tPAtE2 (38 SFU/10^6 cells) vaccinated animals (Fig. 4.9B). The p-tPAtE2IMX313 + pVax group showed the highest responses after splenocyte stimulation with E2 pool 1 (90 SFU/10^6 cells) (Fig. 4.10B), while the ptPAtE1tE2IMX313 + pVax group and the group vaccinated with ptPAtE1IMX313 + ptPAtE2IMX313 showed 5 SFU/10^6 cells and 18 SFU/10^6 cells respectively (Fig. 4.10B). The pVax(empty), p-tPAtE1IMX313 and ptPAtE1tE2IMX313 groups responses were low as expected. A single mouse in the p-tPAtE1IMX313 + pVax group however showed an unexpectedly high response (150 SFU/10^6 cells) following stimulation with E2 pool 1 increasing the overall response for the group to 50 SFU/10^6 cells (Fig. 4.10B).

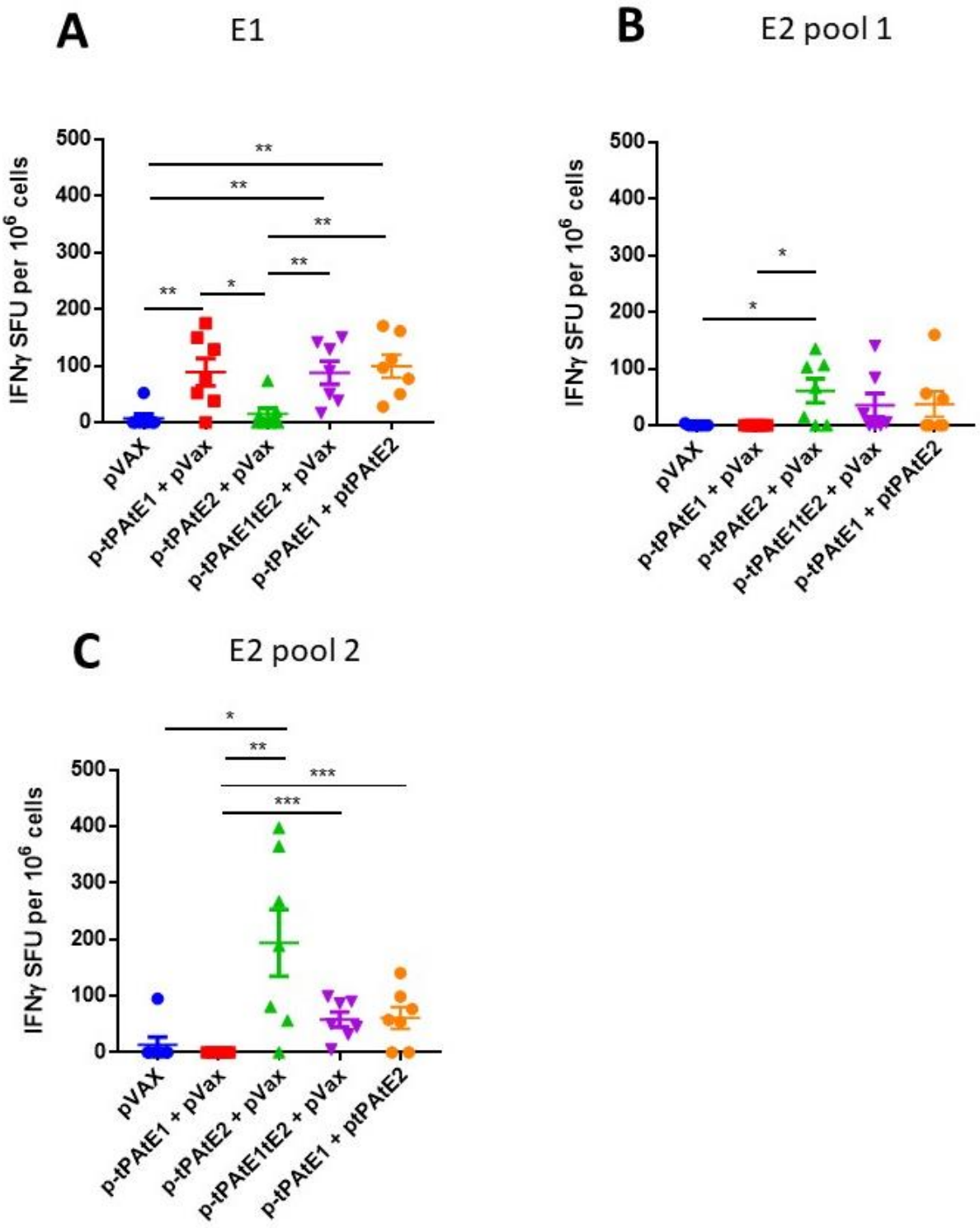


Figure 4.9. HCV-specific cellular immune responses against HCV immunogens in the mice immunised with p-tPAtE1, ptPAtE2 or ptPAtE1tE2. CMI detected by ELISpot. ELISpot assay of splenocytes after stimulation with HCV peptide pools representing E1 and E2. The data are expressed as spot forming units (SFU) per 10^6 cells responses to different peptide pools and presented as the mean ±SEM for seven mice per group. The number of SFU in unstimulated splenocytes was subtracted from the number in peptide-stimulated cells to generate the net HCV response. Mann-Whitney non-parametric t-test was performed and significant p-values between the vaccinated groups are shown. *p<0.05, **p<0.01. Note that the Y-axes may differ.

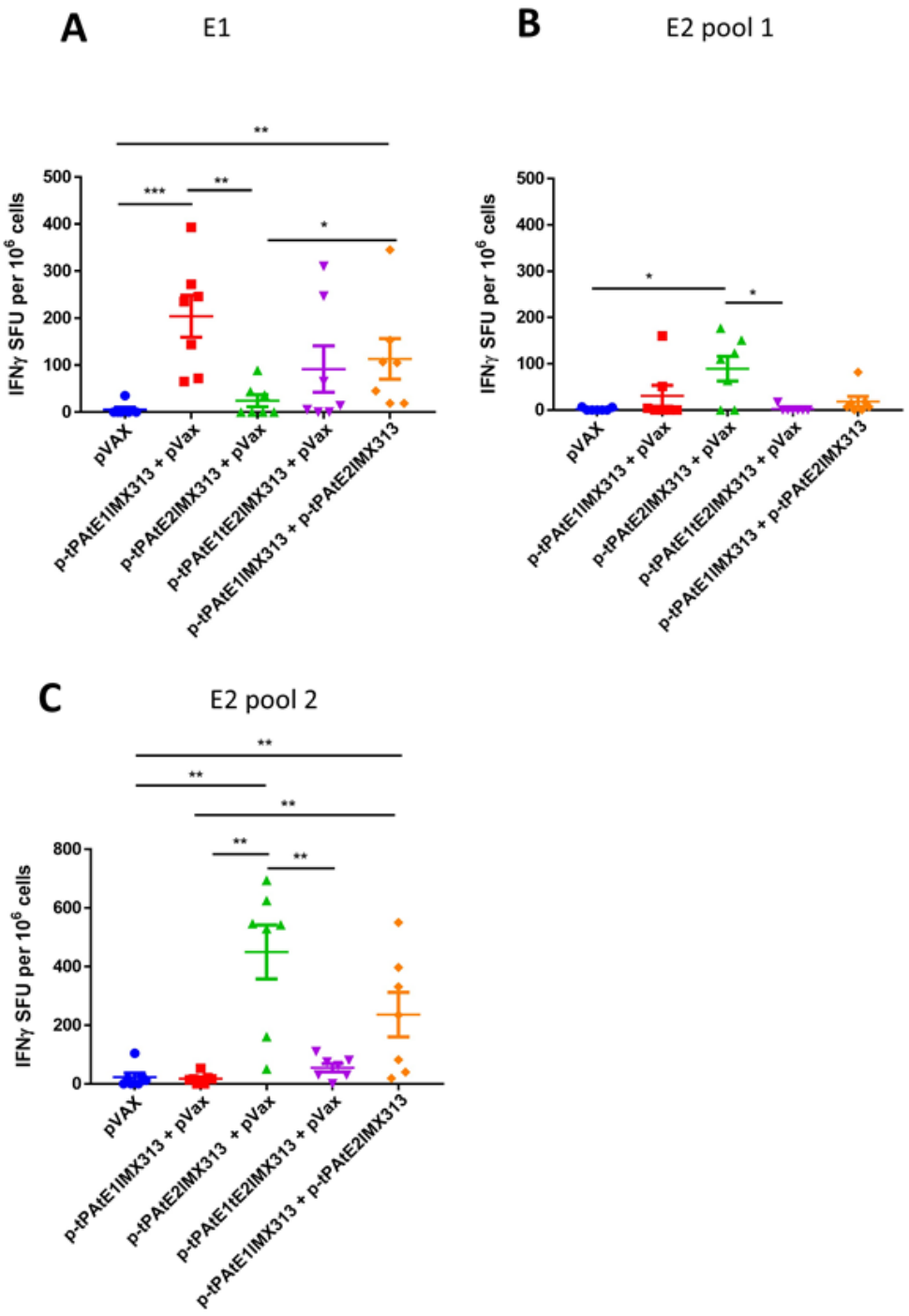


Figure 4.10. HCV-specific cellular immune responses against HCV immunogens in the ptPAtE1IMX313, ptPAtE2IMX313 and ptPAtE1tE2IMX313 immunisation groups. CMI detected by ELISpot. ELISpot assay of splenocytes after stimulation with HCV peptide pools representing E1 and E2. The data are expressed as spot forming units (SFU) per 10^6 cells responses to different peptide pools and presented as the mean ±SEM for seven mice per group. The number of SFU in unstimulated splenocytes was subtracted from the number in peptide-stimulated cells to generate the net HCV response. Mann-Whitney non-parametric t-test was performed and significant p-values between the vaccinated groups are shown. *p<0.05, **p<0.01. Note that the Y-axes may differ.

Vaccination with p-tPAtE2 + pVax or p-tPAtE1 + p-tPAtE2 resulted in significant IFNγ responses to E2 pool 2 peptides (195 SFU/10^6 cells and 63 SFU/10^6 cells respectively) (Fig. 4.9C). The highest response was detected in mice immunised with ptPAtE2IMX313 + pVax with an overall response of 451 SFU/10^6 cells, followed by p-tPAtE1IMX313 + p-tPAtE2IMX313 group (240 SFU/10^6 cells) and p-tPAtE1tE2IMX313 + pVax groups (58 SFU/10^6 cells), while pVax(empty) and p-tPAtE1IMX313 + pVax responses were lower as expected. These results indicate that the secreted E1/E2-IMX313 vaccine strategy can efficiently stimulate CMI responses to HCV E1 and E2.

4.4 Discussion

As described in the introduction in this chapter, E1 and E2 were selected as the vaccine immunogens of choice as they are presented on the surface of the virion and are clearly target for NAb [142, 148, 149, 467, 468]. However, the ideal mode of processing and presenting these immunogens is yet to be determined. In this chapter, a DNA vaccine strategy based on secreted truncated HCV E1 or E2 glycoproteins (tE1 or tE2 respectively) fused to the IMX313 multimerization domain as a molecular adjuvant was examined, to compare the use of the tE1 and tE2 proteins as separate immunogens with their use as a single tE1tE2 polyprotein, in terms of immunogenetic potential.

HCV E1 and E2 glycoproteins are type I transmembrane proteins anchored in the lipid envelope by C-terminal TMDs [448, 469, 470]. To generate secreted forms of these proteins, their TMDs were removed and a signal peptide sequence of the tissue plasminogen activator (tPA) was inserted as a leader sequence to drive a E1/E2 protein into the cellular secretion pathway. The IMX313 oligomerisation domain was also included downstream of these proteins to facilitate the oligomerisation of the secreted proteins. Plasmid DNA encoding three forms of the fusion proteins were generated (tPA-tE1-IMX313, tPA-tE2-IMX313, and tPA-tE1tE2-IMX313) along with control DNA constructs which did not encode the IMX313 domain. These genes were cloned into pVax and the respective proteins expressed and assessed by immunofluorescence and western blot analysis following transfection of HEK293T cells with the vaccine constructs.

Secreted HCV-envelope proteins were successfully detected in the cell culture media in monomeric and multimeric forms. Under non-reducing conditions protein samples from p-tPAtE1, p-tPAtE2 and p-tPAtE1 migrated as monomers. Faint bands corresponding approximately in molar mass to dimers (~75 kDa for p-tPAtE1 and ~120 kDa for p-tPAtE2 and p-tPAtE2tE2, Fig. 4.6B) were also observed. Supernatant fluid collected from cells transfected with DNA constructs encoding the IMX313 domains showed bands of molecular mass

exceeding 250 kDa suggesting successful oligomerisation of the secreted tE1/tE2-IMX313 complexes.

The titers of the E1E2-specific antibody response were measured in the serum of vaccinated mice. The antibody responses reached a plateau after the fourth immunisation. Vaccination with a cocktail of p-tPAtE1IMX313 + p-tPAtE2IMX313 induced a superior E1E2 serum antibody response compared to vaccination with p-tPAtE1tE2IMX313 + pVax. These antibodies were also able to recognise E1E2 proteins in their native heterodimer form as evident by immunofluorescence of HEK293T cells transfected with p-CE1E2-PRF(DA) encoding full length core, E1 and E2 proteins. Mice immunised with p-tPAtE1 + pVax or p-tPAtE1IMX313 + pVax generated weak antibody responses consistent with previous reports suggesting that E1 is poorly immunogenic [146, 471]. However, vaccination with p-tPAtE1IMX313 in combination with p-tPAtE2IMX313 resulted in an increase in E1E2-specific antibody responses, suggesting a synergistic effect when these two proteins were used as separate immunogens as the response was lower in animals vaccinated with p-tPAtE1tE2IMX313. These results also correlated with previous observation that E1 and E2 are less immunogenic when expressed as a single polyprotein and anti-E1 responses are efficiently induced only when E1 is dissociated from E2 [146, 147]. Although significant anti-E1E2 responses were generated in the p-tPAtE1IMX313 + p-tPAtE2IMX313 group, this response was lower compared to those reported by others [420, 445, 472]. Nonetheless the neutralising ability of these antibodies should be assessed to determine whether DNA vaccination with p-tPAtE1IMX313 + p-tPAtE2IMX313 or p-tPAtE1tE2IMX313 + pVax is an effective means of eliciting NAb responses.

In addition to strong humoral responses, robust CMI responses have been proposed to be essential for clearance of HCV during acute infections in humans and chimpanzees [26, 199, 316, 473]. To determine if vaccination with the DNA constructs generated in this chapter was able to elicit CD8+ T cell responses, CMI responses in splenocytes from vaccinated mice were assessed using IFNγ ELISPOT analysis against HCV peptides. The production of IFNγ from CD8+ T cells is an indication of a Th1 type immunity. The results in this chapter indicate that vaccination with DNA constructs encoding secreted E1/E2 or E1/E2-IMX313 could indeed elicit Th1 type responses against E1 and E2 peptides three weeks after the final immunisation. Mice vaccinated with cocktails of DNA constructs encoding tE1 and/or tE2 fused to IMX313 elicited a stronger CMI response compared to mice vaccinated with a similar construct lacking the oligomerisation domain. Animals vaccinated with p-tPAtE1IMX313 + pVax or p-tPAtE2IMX313 + pVax generated the highest IFNγ responses after stimulation with E1 or E2 peptides respectively. However, no synergistic effect was observed as the IFNγ response was

reduced when these constructs were administered together as a p-tPAtE1IMX313 + p-tPAtE2IMX313 cocktail. Nonetheless, similar to the antibody responses, vaccination with p-tPAtE1IMX313 + p-tPAtE2IMX313 consistently induced superior IFNγ responses compared to immunisation with p-tPAtE1tE2IMX313 + pVax. The reduction in p-tPAtE1tE2IMX313 immunogenicity could possibly be due to failed or partial oligomerisation of the proteins *in vivo* or the masking of immunodominant epitopes as a result of the oligomerisation of the larger tPAtE1tE2IMX313 protein. The E1E2-specific ELISPOT results reported in this chapter compared well to those reported in other studies [424, 443, 445], indicating that the E1/E2-IMX313 DNA vaccine is effective in inducing CMI responses even when compared with VLPs or viral vectors.

4.5 Conclusion

The results presented in this chapter demonstrate the successful construction of DNA vaccines encoding secreted forms of HCV envelope proteins fused to the multimerization domain IMX313. Following intradermal DNA vaccination, constructs encoding IMX313 (and thus able to oligomerise) generated superior antibody and CMI responses compared to their monomeric, non-oligomerised counterpart. Immunisation with p-tPAtE1IMX313 + p-tPAtE2IMX313 cocktail was more effective at inducing both antibody and CMI immune responses compared to immunisation with p-tPAtE1tE2IMX313 + p-Vax. The antibody responses describe here will need to be further investigated to assess their neutralising ability. To further increase the E1E2 antibody titer, additional vaccine formulations encoding E1/E2 fused to a modified version of the IMX313 were generated and these results are described in chapter 5.

Chapter 5. The immunogenicity of DNA vaccine encoding secreted HCV-E1/E2 coupled to IMX313P

5.1 Introduction

Neutralising antibodies and cellular immune response both play essential roles in the clearance of HCV infection. The envelope proteins E1 and E2 are major targets for the production of NAbs against HCV. Authentic antigen conformation and repetitive antigenic structures are essential features to induce NAbs [457, 474]. DNA vaccines possess various advantages compared to conventional recombinant protein of peptide immunogens [1, 475]. However DNA vaccines have been reported to have relatively poor immunogenicity, particularly in larger animals, and therefore require adjuvants for maximum effect [475]. Co-administration or co-expression of DNA vaccine along with a plasmid encoding immunomodulatory proteins, such as IL-12, is a popular strategy employed to enhance antigen-specific immune responses [266, 475]. As illustrated previously, genetic adjuvants such as PRF can also boost the host immune response after DNA vaccination [414, 418, 476].

A series of oligomerisation domains from the complement inhibitor C4b-binding protein (C4bp) have been shown to act as adjuvants and IMX313 was shown to induce the highest antigen-specific humoral and cell mediated immune responses of all the domains tested [426, 461-463, 466]. Chapter 4 demonstrated the production of DNA vaccine constructs encoding E1/E2 fused to the oligomerisation domain IMX313. The titers of anti-E1E2 specific antibodies from mice immunised with constructs encoding E1/E2 fused to IMX313 were significantly higher than those from mice immunised with constructs encoding E1/E2 and lacking the IMX313. Moreover, immunisation with these constructs increased E1/E2-specific IFNγ secretion by lymphocytes upon *in vitro* stimulation with E1 and E2 peptides compared to immunisation with constructs without the IMX313 domain. Taken together, these results imply that fusing secreted E1/E2 to the IMX313 domain is an effective strategy to enhance E1E2-specific humoral and cellular immune response. However, higher magnitude immune responses will likely be required to achieve useful cross-protective efficacy. In this chapter, new HCV DNA vaccines encoding E1/E2 antigens fused to a modified oligomerisation domain from the chicken complement inhibitor C4bp termed IMX313P (E1/E2-IMX313P), which was developed by our commercial collaborator simultaneously with the experiments performed in Chapter 4, were generated. The mechanism by which IMX313P enhances immunogenicity is still not well understood. However, similarly to IMX313, oligomerisation may be an important element in IMX313P adjuvanticity [461, 464]. The efficacy of these DNA vaccines in eliciting humoral and cellular immunity in mice was evaluated.

5.2 Aims

The aims of this chapter are to:

1. Generate DNA vaccine constructs encoding HCV-envelope proteins fused to the IMX313P oligomerisation domain.
2. Confirm HCV-envelope protein expression and IMX313P oligomerisation.
3. Assess the immunogenicity of these constructs in vivo

5.3 Results

5.3.1 Plasmid construction

To generate the plasmid encoding secreted forms of E1 and E2 fused to the IMX313P domain (Fig. 5.1A), tPA fwd and IMX313P rev primers (appendix I) were used to generate individual PCR fragments encoding tPAtE1IMX313P, tPAtE2IMX313P, tPAtE1tE2IMX313P using the p-tPAtE1IMX313, p-tPAtE2IMX313, p-tPAtE1tE2IMX313 plasmids generated in chapter 4 as templates, respectively. These fragments were digested using the restriction enzymes *EcoRI* and *NheI* and then ligated into pVax previously digested with the same restriction enzymes. *E.coli* (DH5α) cells were transformed using the ligation mix and colony PCR was performed to identify clones. Positive clones were further analysed using restriction enzyme digestion and the correct sequences of the positive clones were confirmed by DNA sequencing (Chapter 2 – section 2.2.2.13). Maps of the constructs generated in this chapter are shown in Figure 5.1B.

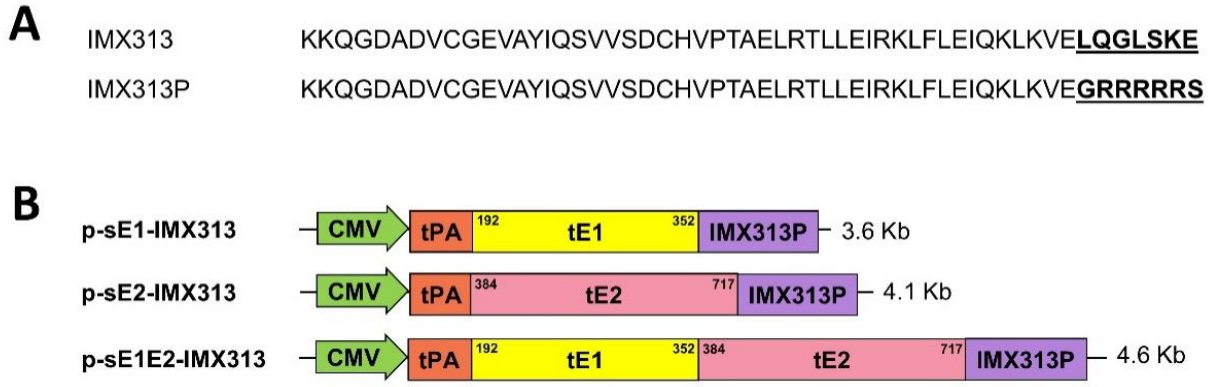


Figure 5.1. C4bp oligomerization domains and plasmid maps of DNA constructs used in this chapter. A) Alignment of the amino acid sequences of C4bp oligomerization domains IMX313 and IMX313P. Differences in amino acid sequence are underlined in bold. B) DNA constructs produced by fusing the IMX313P oligomerisation domain to the 3' end of the genes encoding secreted tE1 or tE2 proteins. The numbering corresponds to the amino acid position in the HCV polyprotein. DNA plasmid sizes are shown on the right.

5.3.2 Protein expression and oligomerisation

Immunofluorescence analysis was conducted using anti- HCV (serum from HCV-infected patients) to assess the expression of E1 and E2 in HEK293T cells transfected with p-

tPAtE1IMX313P, p-tPAtE2IMX313P or p-tPAtE1tE2IMX313P. As expected positive fluorescence was observed in cells transfected with all DNA constructs, but not in cells transfected with the pVax control (Fig. 5.2 – top far-left panel). Cells transfected with the same DNA constructs were stained with normal human serum (anti-NHS) as control for non-specific background staining (Fig. 5.2 - bottom panels).

The oligomeric state of the E1/E2-IMX313P fusion proteins in purified and concentrated cell culture media collected from HEK293T cells transfected with p-tPAtE1IMX313P, p-tPAtE2IMX313P or p-tPAtE1tE2IMX313P was investigated by western blot analysis under reducing (Fig. 5.3A) and non-reducing (Fig. 5.3B) conditions. Each of the proteins migrated as monomers under reducing conditions with molecular weight of ~35 KDa for E1 and ~68 KDa for E2 (Fig. 5.3A). The HCV envelope-IMX313P fusion proteins migrated under non-reducing conditions as a broad oligomeric band of molecular mass greater than 250 kDa. These high molecular mass species were not observed in supernatant from cells transfected p-tPAtE1, p-tPAtE2 or p-tPAtE2tE2 (Fig. 5.3B). These proteins migrated, under non-reducing conditions, as monomers of approximately ~50 kDa for E1 and ~80 kDa for E2. Bands of approximately ~75kDa for p-tPAtE1 and ~120 kDa for p-tPAtE2 and p-tPAtE1tE2 corresponding to protein dimers were also observed (Fig. 5.3B). No HCV-specific bands were detected in pVax control and cell only samples. These data suggest that p-tPAtE1IMX313P, p-tPAtE2IMX313P or p-tPAtE1tE2IMX313P transfection results in successful expression and formation of protein species of molecular mass greater than 250 kDa.

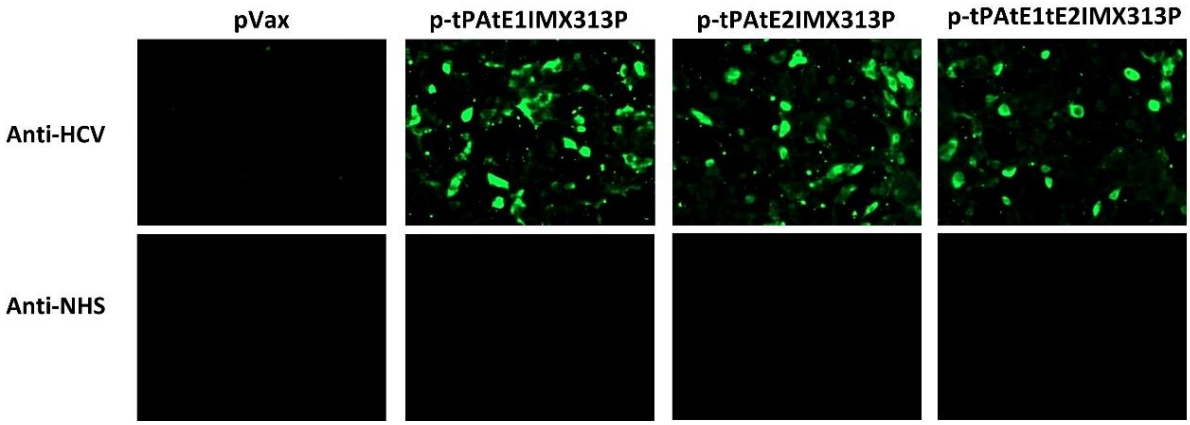


Figure 5.2. Immunofluorescence of HCV envelope proteins in HEK293T cells. Fluorescence staining of HEK293T cells transfected p-tPAtE1IMX313P, p-tPAtE2IMX313P or p-tPAtE1tE2IMX313P. Approximately 48 hours post transfection cells were fixed/permeabilised and stained with Anti-HCV (pooled HCV genotype1 patient sera, green) and anti-NHS (normal human serum) as a control for cross reaction and unspecific background. pVax transfected cells were used as control non-specific fluorescence (magnification: 400×).

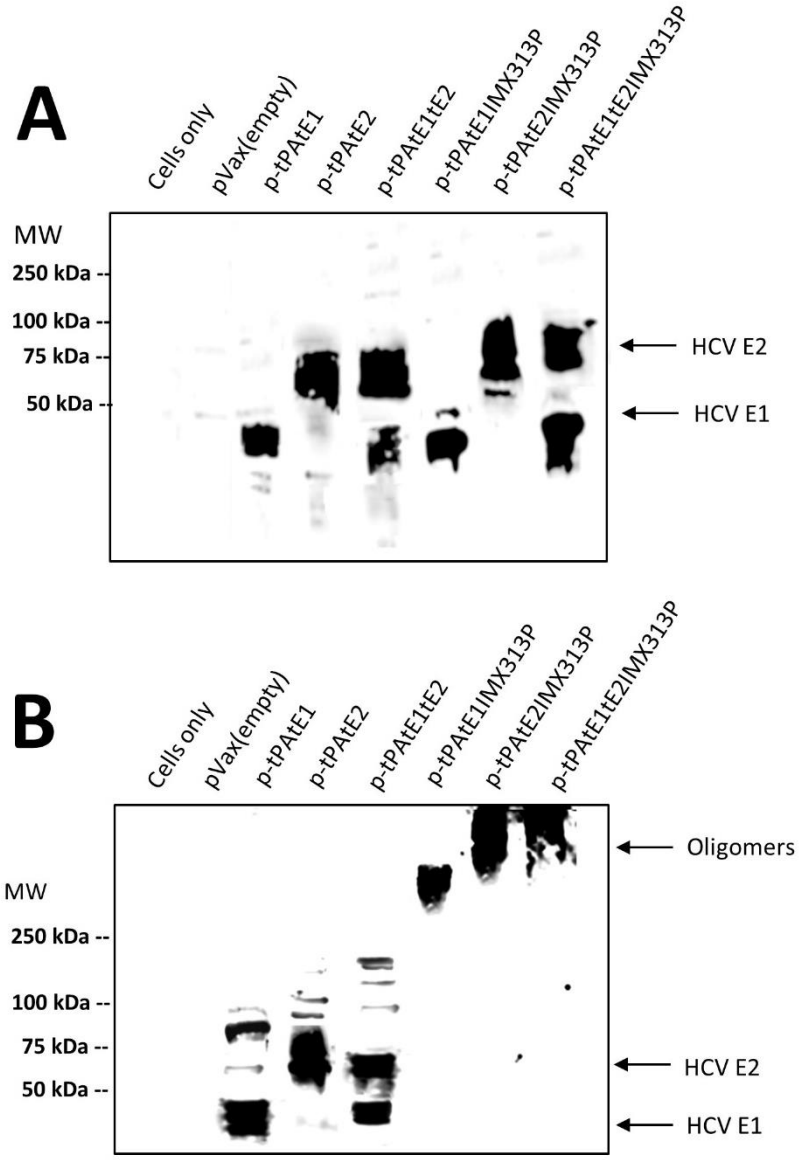


Figure 5.3. Expression and oligomerisation of HCV envelope proteins from the DNA vaccines. HEK293T cells were transfected with p-tPAtE1, p-tPAtE2, p-tPAtE1tE2, p-tPAtE1IMX313P, p-tPAtE2IMX313P or p-tPAtE1tE2IMX313P. At 72 hours post transfection, cell culture media was harvested, centrifuged to remove detached cells then filtered through a 0.22 µm filter to remove remaining cell debris. Clarified supernatants were concentrated by ultrafiltration through a 70,000-molecular-weight cut off filter (70K MWCO; Amicon). The protein samples were then subjected to SDS-PAGE and immunoblotting under (A) reduced and (B) non-reduced conditions, and stained with anti-HCV antibodies. Molecular masses (in kDa) of protein molecular weight (MW) markers are indicated on the left; HCV-specific proteins are indicated on the right. The untransfected cells and pVax-transfected cells represent controls to show non-specific bands.

5.3.3 Animal vaccinations

Female Balb/C mice, aged 6-8 weeks old were grouped into 7 mice per group. Each group was administered pVax, or a combination of pVax with p-tPAtE1IMX313P, p-tPAtE2IMX313P or p-tPAtE1tE2IMX313P (Fig. 5.4A). The remaining mice received a combination of p-tPAtE1IMX313P and p-tPAtE2IMX313P. Mice were immunised 6 times at 3 week intervals via the intradermal route in the ear pinnae (Fig. 5.4B). Each group of animals was vaccinated with a total of 20.65 picomoles per dose per animal. The vaccine dosage was determined using 50µg DNA of the p-tPAtE1 as the baseline molecular mass for vaccinations. Blood was collected on a weekly basis and the serum analysed for the presence of anti-E1/E2 antibodies. Mice were sacrificed 21 days after the final vaccination, the spleen removed and prepared for IFN-γ ELISPOT analysis.

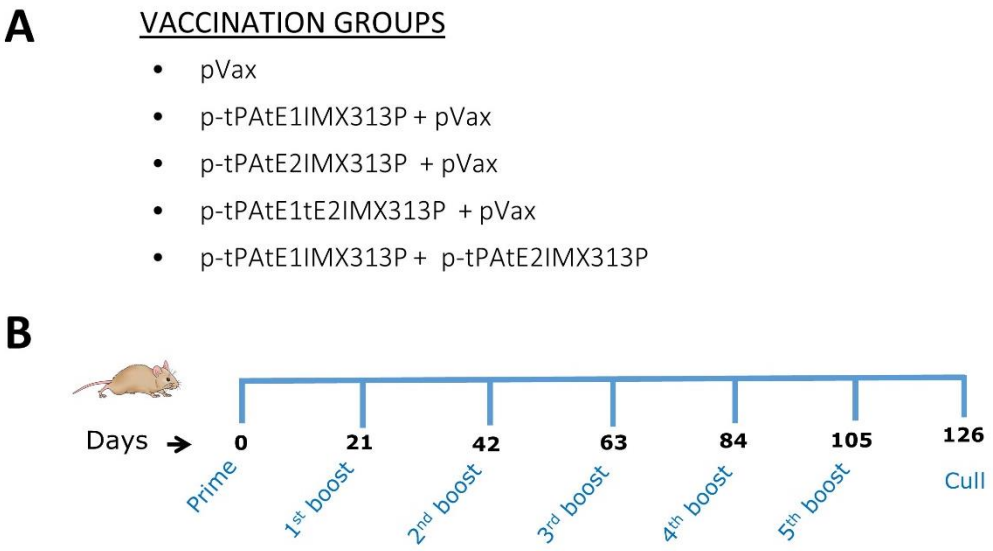


Figure 5.4. Vaccination in 6-8 weeks old female Balb/C mice. Vaccination groups (A) and immunisation schedule of animals (B). Female Balb/C mice (n= 7/group) were inoculated intradermally with 20.65 picomoles of the respective plasmids at three weekly intervals. pVax was included in the vaccine cocktail to ensure that all animals were vaccinated with an equimolar amount of DNA. Blood was collected weekly and spleens were harvested 21 days post the final vaccination.

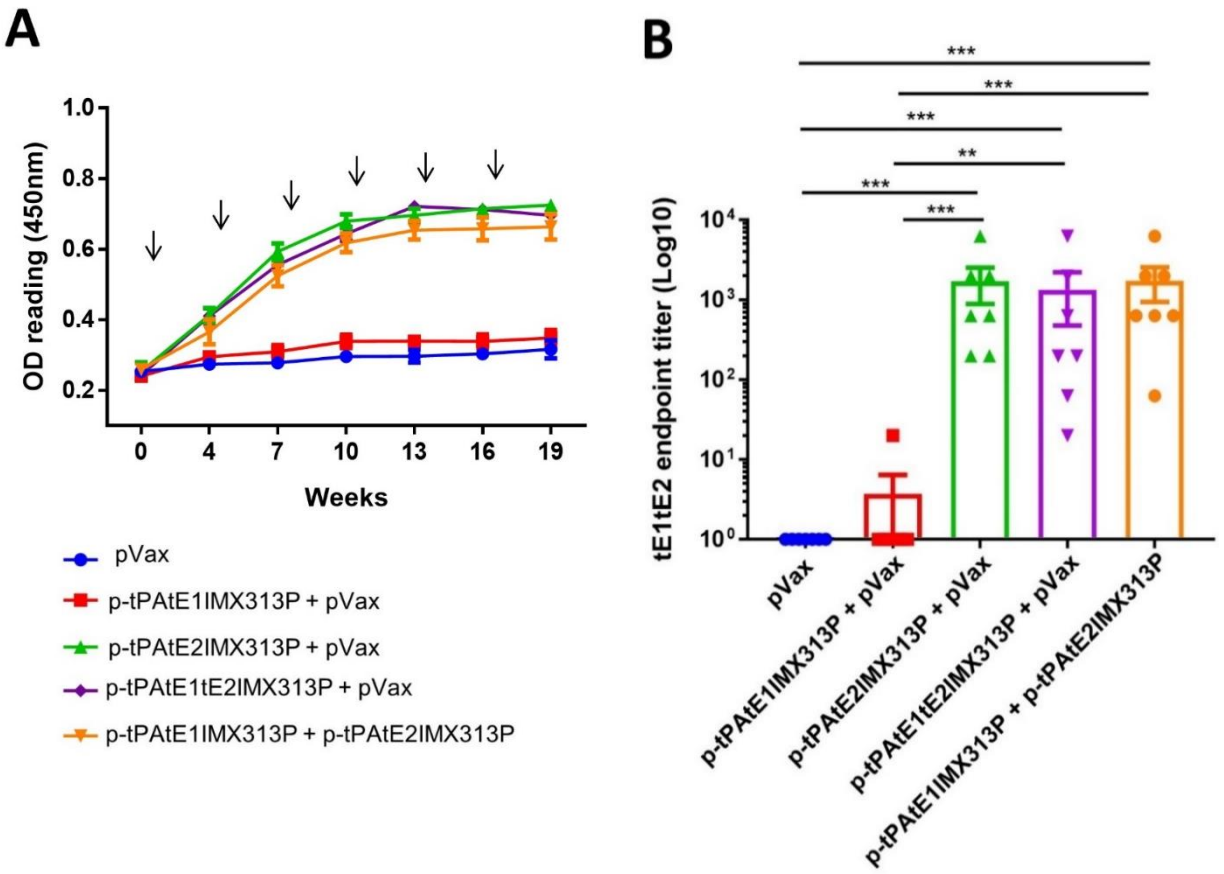
5.3.4 Immune responses

5.3.4.1 Humoral responses

To determine the antibody responses elicited following vaccination with the DNA vaccine encoding secreted E1/E2-IMX313P fusion protein, mouse serum was collected after each vaccination and analysed for E1E2-specific antibodies by ELISA.

As shown in Figure 5.5A, the antibody levels peaked and plateaued following the 3rd booster immunisation. Antibody titers measured 3 weeks post the final vaccination showed that

immunisation with a cocktail of p-tPAtE1IMX313P + p-tPAtE2IMX313P resulted in the highest anti-E1E2 antibody response with an average titer of 1/1749, followed by p-tPAtE2IMX313P + pVax-vaccinated mice (average titer 1/1707), p-tPAtE1tE2IMX313P + pVax (average titer 1/1346) and p-tPAtE1IMX313P + pVax (average titer 1/2.5) (Fig. 5.5B). These antibodies were also capable of binding to the E1E2 heterodimer in an immunofluorescence analysis (Fig. 5.5C). Moreover, the E1E2-specific antibody titers induced following immunisation with these constructs were significantly higher when compared to titers induced following immunisation with constructs encoding E1/E2 fused to IMX313 (Fig 5.5D, see Chapter 4 – section 4.3.4.1). Collectively, the data indicate that DNA vaccines encoding E1/E2-IMX313P are able to elicit robust HCV E1E2-specific antibody responses and this response was superior to that induced by immunisation with DNA vaccines encoding E1/E2-IMX313.



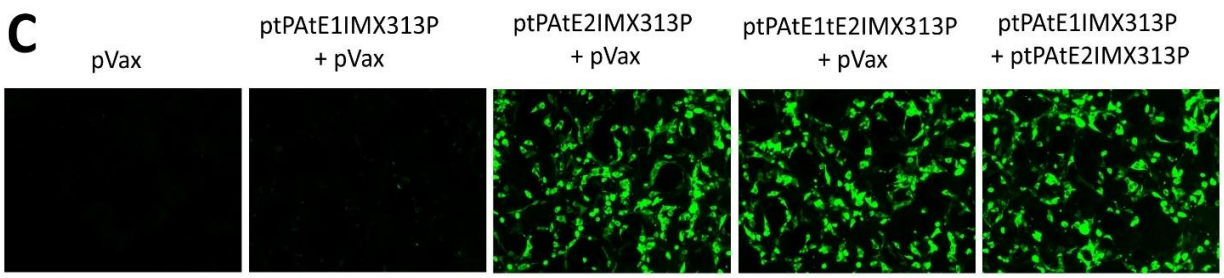


Figure 5.5. Induction of anti-E1E2 antibodies following vaccination with p-tPAE1IMX313P, p-tPAtE2IMX313P or p-tPAtE1tE2IMX313P. A) Serum antibody response specific for HCV E1E2 proteins. Serum from vaccinated mice was diluted (1/50) and anti-E1E2-specific antibodies were measured by ELISA at the indicated time points. Black arrows indicated when the vaccine was administered. Values represent mean responses in each group (n = 7) ± SEM. B) Serum from individual mice (3 weeks after the final immunisation) was serially diluted, and endpoint titers for immunised mouse sera were calculated. Values for individual mice are shown (n = 7) and bars represent the mean ± SEM. Mann-Whitney non-parametric t-test was performed and significant p-values between the vaccinated groups are shown. *p<0.05, **p<0.01, *** p < 0.001. C) Immunofluorescent analysis of the presence of antibodies specific for full-length E1 and E2 proteins in immune mouse sera (magnification 200×). HEK293T were transfected with p-CE1E2-PRF(DA) encoding full length E1E2 proteins and pooled sera from mice immunised with p-tPAE1IMX313P + pVax, p-tPAtE2IMX313P + pvax, p-tPAtE1tE2IMX313P + pVax or p-tPAE1IMX313P + p-tPAtE2IMX313P were used as the primary antibody at a 1:50 dilution.

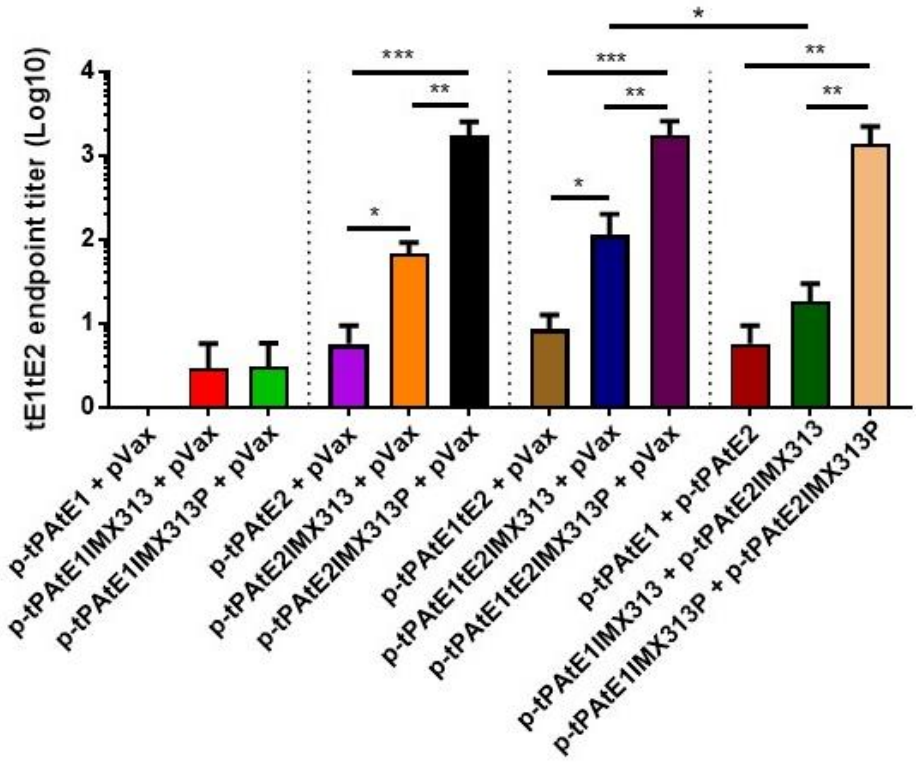


Figure 5.6. Induction of anti-E1E2 antibodies following vaccination with DNA construct encoding E1/E2 fused to IMX313 or IMX313P. Serum from individual mice (3 weeks after the final immunisation) was serially diluted, and endpoint titers for immunised mouse sera were calculated. Values for individual mice are shown (n = 7) and bars represent the mean ± SEM. All experiments were performed simultaneously. Mann-Whitney non-parametric t-test was performed and significant p-values between the vaccinated groups are shown. *p<0.05, **p<0.01, *** p < 0.001.

5.3.4.2 Cell mediated immune responses

IFNγ ELISPOT was conducted to assess the induction of cellular immune responses against the E1 and E2 proteins. Two weeks following the final dose, splenocytes were harvested and stimulated with HCV peptide pools representing E1 or E2 proteins. When stimulated with E1 peptide, mice vaccinated with ptPAtE1tE2IMX313P + pVax showed the most IFN-γ-secreting T cells with an average of 580.5 SFU/10^6 cells. Mice that received ptPAtE1IMX313P + pVax showed an average of 380.5 SFU/10^6 cells followed by and ptPAtE1IMX313P + ptPAtE2IMX313P (mean 242.8 SFU/10^6 cells) (Fig. 5.7A). Both pVax(empty) or ptPAtE2IMX313 + pVax vaccination groups showed significantly lower IFNγ responses as expected.

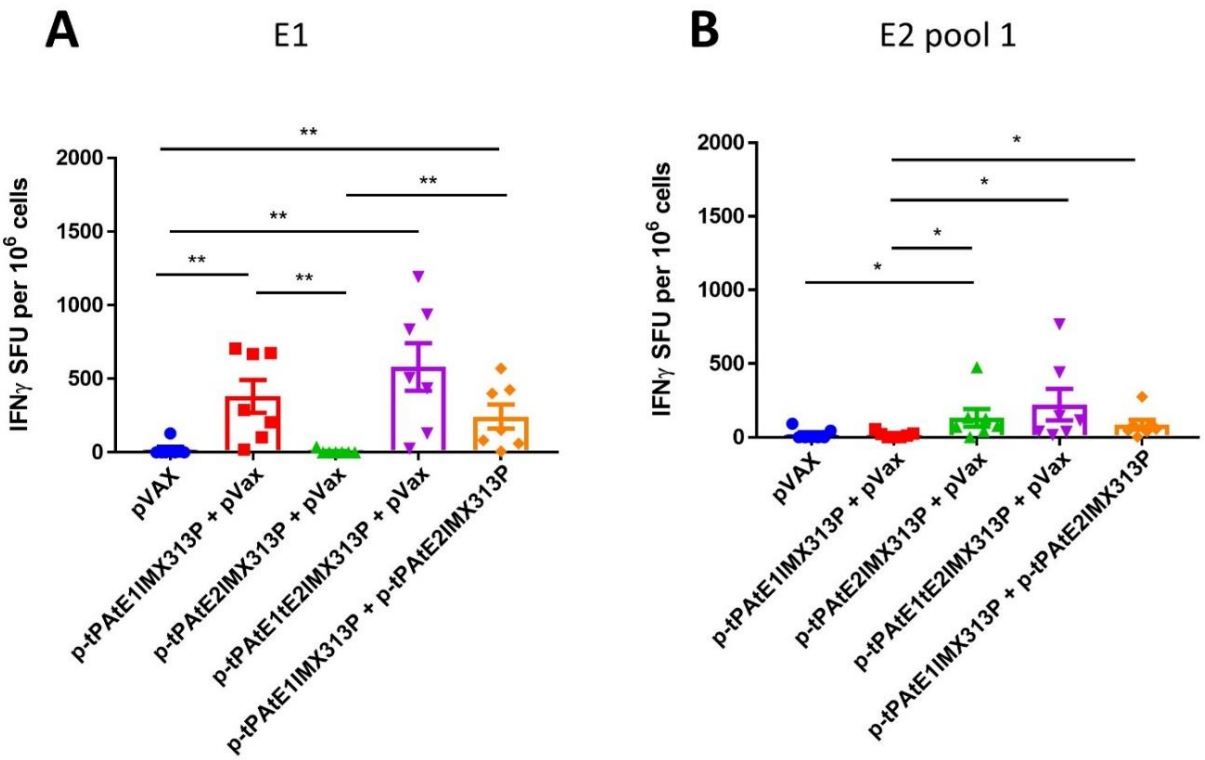
After stimulation of splenocytes with the E2 pool 1 peptides, significant IFN-γ secretion was detected in p-tPAtE1tE2IMX313P + pVax (222.7 SFU/10^6 cells), p-tPAtE2IMX313P + pVax (132.5 SFU/10^6 cells) and p-tPAtE2IMX313P + p-tPAtE2IMX313P (86.1 SFU/10^6 cells) vaccinated animals (Fig. 5.7B). The pVax, p-tPAtE1IMX313P + pVax groups were low as expected with 19.7 SFU/10^6 cells and 16.2 SFU/10^6 cells respectively (Fig. 5.7B).

The highest response following splenocytes stimulation with E2 pool 2 peptides was detected in mice immunised with p-tPAtE2IMX313P + pVax with an overall response of 1049.5 SFU/10^6 cells (Fig. 5.7C). Immunisation with p-tPAtE1IMX313P + p-tPAtE2IMX313P induced 972.5 SFU/10^6 cells followed by p-tPAtE1tE2IMX313P + pVax immunised group (645 SFU/106 cells). While pVax and p-tPAtE1IMX313P + pVax responses were lower as expected. Overall these results indicate that the secreted E1/E2-IMX313P vaccine strategy can efficiently stimulate CMI responses to HCV E1 and E2.

The HCV-specific cellular immune responses results shown here were expressed as fold increase over the pVax control group and compared to the data reported in chapter 4 section 4.3.4.2 (Fig. 5.8). E1-stimulated cells from mice vaccinated with DNA constructs encoding IMX313P induced higher INFγ release with a 42.7 INFγ SFU fold increase (relative to pVax control) for p-tPAtE1tE2IMX313P + pVax vaccinated mice compared to 6.7 INFγ SFU fold increase for p-tPAtE1tE2IMX313 + pVax (Fig. 5.8A). p-tPAtE1IMX313P + p-tPAtE2IMX313P vaccinated mice showed a 17.8 INFγ SFU fold increase of IFNγ-secreting cells compared to mice vaccinated with p-tPAtE1IMX313 + p-tPAtE2IMX313 that showed a 8.3 INFγ SFU fold increase (Fig. 5.8A).

A similar trend was observed in E2 pool 1-stimulated cells in which p-tPAtE1tE2IMX313P + pVax and p-tPAtE1IMX313P + p-tPAtE2IMX313P vaccinated mice induced a higher response (30.17 INFγ SFU fold increase for p-tPAtE1tE2IMX313P + pVax and 11.6 INFγ SFU fold increase for p-tPAtE1IMX313P + p-tPAtE2IMX313P) compared to mice immunised with p-tPAtE1tE2IMX313 + pVax (0.3 INFγ SFU fold increase) or p-tPAtE1IMX313 + p-tPAtE2IMX313 (2.5 INFγ SFU fold increase) (Fig. 5.8B).

As shown in Figure 5.8C, following stimulation with E2 pool 2 peptides, a 15.4 INFγ SFU fold increase was observed in the p-tPAtE1tE2IMX313P + pVax group as opposed to a 1.32 INFγ SFU fold increase in mice immunised with p-tPAtE1tE2IMX313 + pVax (Fig. 5.8C). p-tPAtE1IMX313P + p-tPAtE2IMX313P vaccinated mice showed a 23.3 INFγ SFU fold increase compared to 5.6 INFγ SFU fold increase for mice vaccinated with p-tPAtE1IMX313 + p-tPAtE2IMX313 (Fig. 5.8C). Overall, the results shown in Figure 5.8 revealed that vaccination with a DNA construct encoding E1/E2 proteins fused to the IMX313P domain induced superior cell mediated immune responses when compared to vaccination with DNA construction encoding E1/E2 proteins fused to the IMX313 domain.



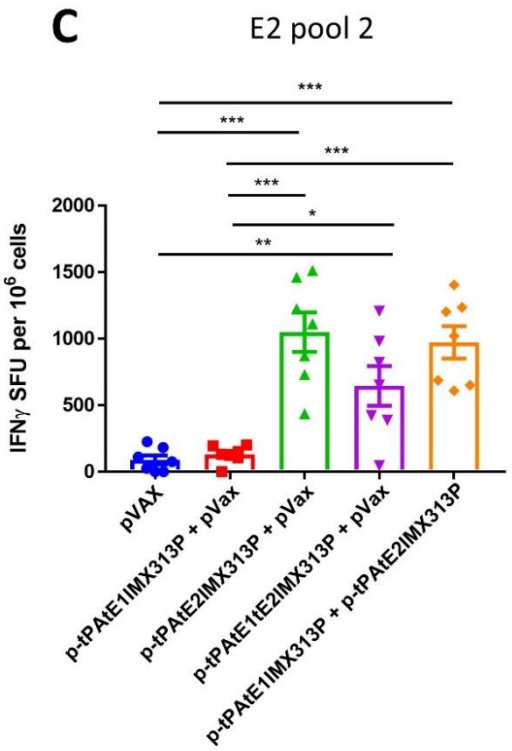


Figure 5.7. HCV-specific cellular immune responses against HCV immunogens in the mice immunised with p-tPAtE1IMX313P, ptPAtE2IMX313P or ptPAtE1tE2IMX313P. CMI detected by ELISpot of splenocytes after stimulation with HCV peptide pools representing (A) E1 and (B & C) E2. The data are expressed as spot forming units (SFU) per 10^6 cells responses to different peptide pools and presented as the mean ±SEM for seven mice per group. The number of SFU in unstimulated splenocytes was subtracted from the number in peptide-stimulated cells to generate the net HCV response. Mann-Whitney non-parametric t-test was performed and significant p-values between the vaccinated groups are shown. *p<0.05, **p<0.01, *** p<0.001. Note that the Y-axes may differ.

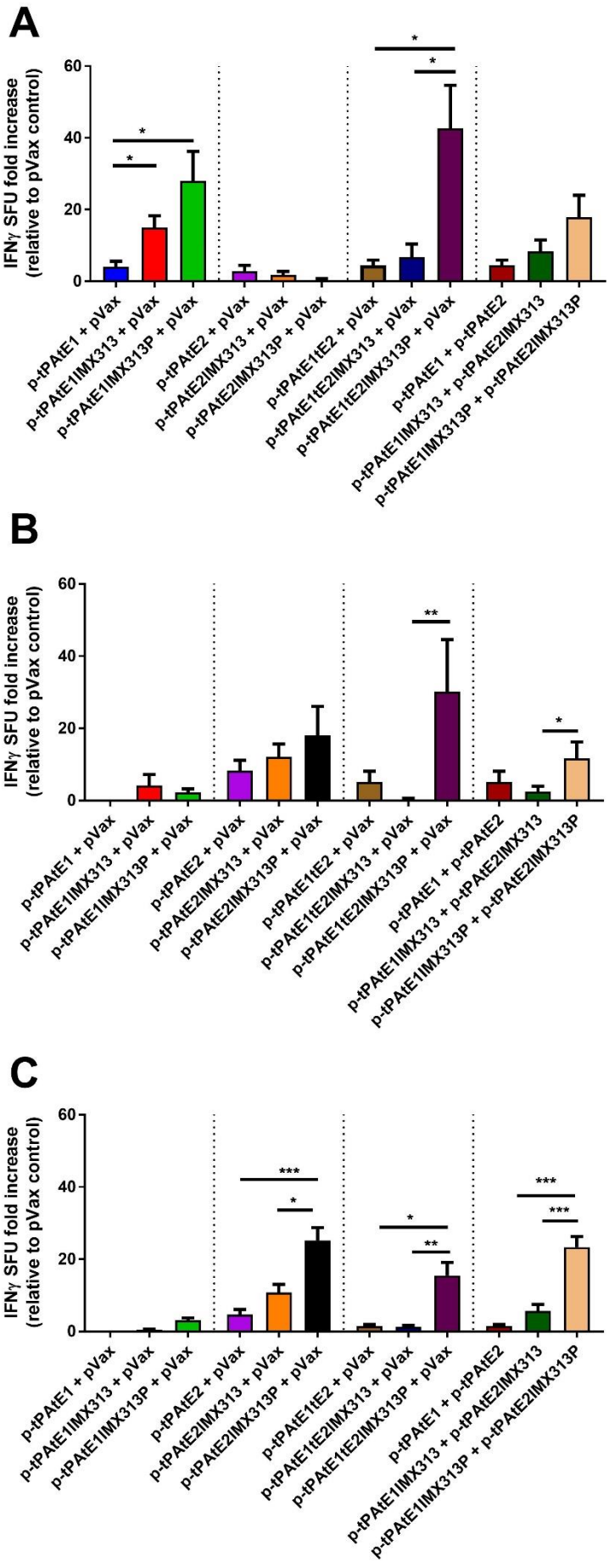

A
B
C
IFNγ SFU fold increase (relative to pVax control)
60
40
20
0
p-tPAtE1 + pVax
p-tPAtE1IMX313 + pVax
p-tPAtE1IMX313P + pVax
p-tPAtE2 + pVax
p-tPAtE2IMX313 + pVax
p-tPAtE2IMX313P + pVax
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Figure 5.8. HCV-specific cellular immune responses in mice immunised with DNA vaccines encoding E1/E2 fused to IMX313 or IMX313P domains. IFNγ response detected by ELISpot of splenocytes after stimulation with (A) E1, (B) E2 pool 1 and (C) E2 pool 2 peptide pools. Data are plotted as mean (n = 7) SFU fold increase (+SEM) relative to pVax control. Mann-Whitney non-parametric t-test was performed and significant p-values between the vaccinated groups are shown. *p<0.05, **p<0.01, *** p < 0.001.

5.4 Discussion

This chapter describes a DNA vaccine strategy based on secreted truncated tE1 or tE2 fused to the IMX313P oligomerisation domain as a molecular adjuvant. The IMX313P oligomerisation domain was introduced downstream of the tE1/tE2 proteins to generate p-tPAtE1IMX313P, p-tPAtE2IMX313P and p-tPAtE1tE2IMX313P. Successful E1/E2 protein expression was evaluated by immunofluorescence and oligomerisation of the proteins was evaluated using western blot analysis following transfection of HEK293T cells with the vaccine constructs.

E1/E2 proteins were successfully detected in HEK293T cells transfected with the vaccine constructs using immunofluorescence using anti-HCV-positive human serum. Western blot analysis demonstrated that secreted HCV envelope proteins were successfully detected in the cell culture media in monomeric and multimeric forms. Under non-reducing conditions, supernatant collected from cells transfected with p-tPAtE1IMX313P, p-tPAtE2IMX313P or p-tPAtE1tE2IMX313P showed bands of molecular mass exceeding 250 kDa while protein samples from p-tPAtE1, p-tPAtE2 and p-tPAtE1 migrated as monomers. Under non-reducing conditions, bands corresponding approximately in molar mass to dimers (~75 kDa for p-tPAtE1 and ~120 kDa for p-tPAtE2 and ~170 kDa for p-tPAtE2tE2, Fig. 5.3B) were also observed. Overall these results suggest the successful oligomerisation of the secreted tE1/tE2-IMX313P protein complexes.

The levels of tE1tE2-specific antibodies in vaccinated mice were measured using ELISA. The antibody response peaked after the fourth immunisation in all the vaccination groups apart from pVax and p-tPAtE1IMX313P + pVax vaccinated mice. Vaccination with a cocktail of p-tPAtE1IMX313P + p-tPAtE2IMX313P induced a superior tE1tE2 serum antibody response compared to vaccination with p-tPAtE1tE2IMX313P + pVax. The ELISA results were also validated using immunofluorescence and showed that these antibodies were also able to recognise E1E2 proteins in their native heterodimer form (Fig. 5.5C). Mice immunised with p-tPAtE1IMX313P + pVax generated weak antibody responses consistent with results obtained in chapter 4 (Fig. 4.7 & Fig 4.8), suggesting that E1 could be weakly immunogenic [146, 471]. However, a synergistic effect was observed when mice were vaccinated with p-

tPAtE1IMX313P in combination with p-tPAtE2IMX313P resulting in an increase in E1E2-specific antibody responses. Using recombinant E1 or E2 proteins separately to coat wells in ELISA would clarify whether vaccination with p-tPAtE1IMX313P + p-tPAtE2IMX313P or p-tPAtE1tE2IMX313P + pVax induced predominantly anti-E2 antibodies, or a combination of both anti-E1 or anti-E2 antibodies. Nevertheless, the antibody responses generated by vaccination with these constructs were comparable to those reported in the literature [420, 445, 472]. Further studies are required to determine the functional activity of these antibodies.

To determine the production of IFNγ from CD8+ T cells following vaccination with the DNA constructs, IFNγ ELISPOT analysis against HCV peptides was conducted. Animals vaccinated with p-tPAtE1tE2IMX313P + pVax generated the highest IFNγ responses after stimulation with E1 peptide or E2 peptide pool 1, while p-tPAtE2IMX313P + pVax generated the highest IFNγ responses when stimulated with the E2 peptide pool 2. No significant differences in IFNγ responses were observed between p-tPAtE1IMX313P + p-tPAtE2IMX313P and p-tPAtE1tE2IMX313P + pVax vaccinated mice, following stimulation with E1 or E2 peptides. The E1E2-specific ELISPOT results reported in this chapter compared well to those reported in other studies [424, 443, 445], indicating that the E1/E2-IMX313P DNA vaccine is effective in inducing CMI responses.

Fusion of E1/E2 to IMX313P resulted in significantly higher tE1tE2 antibody and cell mediated immune responses compared to fusion of the same proteins to IMX313. Interestingly, unlike fusion of E1/E2 to IMX313 which showed an increase in antibody titer following p-tPAtE1IMX313 + p-tPAtE2IMX313 vaccination (Chapter 4 – section 4.3.4.1), no differences in tE1tE2 antibody responses were observed in animals vaccinated with p-tPAtE1tE2IMX313P + pVax compared to animals vaccinated with p-tPAtE1IMX313P + p-tPAtE2IMX313P. A similar trend was observed in terms of cell mediated immunity suggesting that fusing E1/E2 to IMX313P is a more effective means of enhancing HCV specific responses in animals compared with IMX313.

The significant differences in immunogenicity following fusion of HCV proteins to IMX313 or IM313P suggest that oligomerisation might not be the only important element responsible for the enhanced E1/E2-specfic responses. The difference in immune responses might have been facilitated by different E1/E2 presentation when they fused to IMX313 or IMX313. Other factors such as increased serum half-life, improved antigen uptake and/or prolonged antigen processing might have contributed to the observed effects [462]. The mode of action of the IMX313 and IMX313P domains should be determined to confirm this.

5.5 Conclusion

DNA vaccines encoding secreted E1/E2-IMX313P fusion proteins produced in this chapter generated significant antibody and CMI responses. No significant differences were observed following immunisation with p-tPAtE1IMX313P + p-tPAtE2IMX313P cocktail compared to immunisation with p-tPAtE1tE2IMX313 + pVax. Furthermore, the data demonstrated that E1 and E2 proteins are much more immunogenic when fused to the IMX313P domain as opposed to fusion with IMX313. Therefore, the IMX313P domain represents a more effective adjuvant that enhances the immunogenicity of HCV-E1E2 based DNA vaccines. However, despite the remarkable capacity of these DNA constructs to induce and boost E1/E2-specific humoral and cell mediated immunity, higher frequency responses are likely to be necessary to achieve effective neutralisation and protection. Chapter 6 aims at increasing E1/E2-specific responses generated by DNA constructs encoding E1/E2-IMX313 by boosting with recombinant proteins or HCV-VLPs.

Chapter 6. Improving the immunogenicity of secreted HCV-E1/E2-IMX313P DNA vaccines with a VLP or recombinant E1E2 protein boost

6.1 Introduction

DNA-based vaccination offers a unique alternative method of immunisation. DNA vaccines can induce both humoral and cellular immunity, persistent expression of heterologous antigen, and a memory response against the infectious disease. Additionally, DNA vaccines are safe in humans, and are easy and cost effective to produce on a large-scale [302, 477]. However, DNA vaccines still need to be improved as they induce only poor immune responses in large animals despite being effective in mice [1]. A possible reason for this could be that, compared to mice, the naked DNA vaccine is less efficiently transduced *in vivo* in humans, leading to low antigen expression. Therefore, the introduction of a DNA vaccine encoding HCV-E1/E2-IMX313P into the clinic would likely require a further enhancement of its immunogenicity.

Considerable efforts are being made to improve the immune responses evoked from DNA immunisation, either through addition of various immuno-stimulatory mediators, and /or through formulation and delivery [478-482]. Heterologous prime-boost vaccination is a means of priming the immune system by administration of a target antigen via one type of vector, with subsequent boosting of immunologic memory by re-administration of the antigen in the context of a different vector that optimally confers higher antigen levels than during priming [478, 483, 484]. The DNA prime–protein boost regimen is known to be one of such effective protocols. DNA-based priming vaccination followed by recombinant protein booster immunisation with relevant soluble antigens has been shown to be well tolerated and elicited both cellular and humoral immune responses in HIV and *Plasmodium* infected patients [484-488].

Chapter 5 demonstrated that immunisation with p-tPAtE1IMX313P + p-tPAtE2IMX313P or p-tPAtE1tE2IMX313P + pVax resulted in the induction of E1/E2-specific humoral and cell-mediated immunity. In this chapter, the immunogenicity of this DNA prime/DNA boost approach is compared against DNA prime/protein boost or DNA prime/VLP boost approaches.

6.2 Aims

The major aims of this chapter are to:

4. Evaluate the immunogenicity of DNA prime/DNA boost, DNA prime/E1E2 recombinant protein boost or DNA prime/HCV VLP boost regimens, using DNA encoding E1/E2 fused to IMX313P, E1E2 recombinant protein and HCV VLPs.

5. Assess the neutralising ability of the antibodies generated by the different vaccination regimens.

6.3 Results

6.3.1 Plasmid construction and protein expression.

The p-tPAtE1IMX313P, p-tPAtE2IMX313P, p-tPAtE1tE2IMX313P plasmids (Fig. 6.1) were generated and evaluated as stated in chapter 5 - section 5.3. The secreted tE1tE2 poly-His-tagged fusion proteins were affinity purified and analysed by western blotting as described in chapter 2 section 2.2.3.1 and chapter 3 – section 3.6.3.1. The production and purification of the genotype 1 HCV VLPs (kindly provided by Professor Joseph Torresi) have been described elsewhere [420, 421, 489].

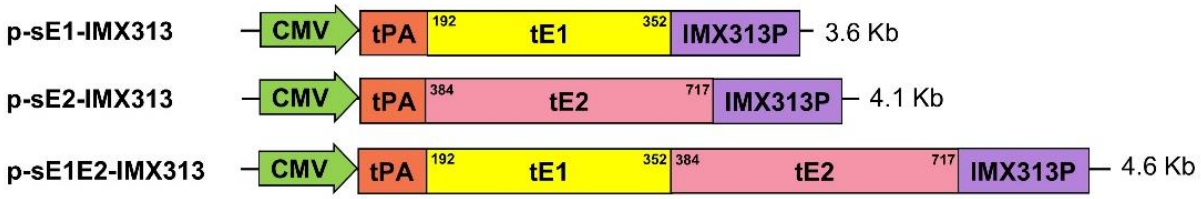


Figure 6.1. Plasmid maps of DNA constructs used in this chapter. A) DNA plasmids encoding secreted, truncated E1 and E2 fused to IMX313P. The numbering corresponds to the amino acid position in the HCV polyprotein. DNA plasmid sizes are shown on the right.

6.3.2 Animal vaccinations

Female Balb/C mice, aged 6-8 weeks old were grouped into 7 mice per group. One group was immunised with pVax, three groups were immunised with combinations of p-tPAtE1IMX313P and p-tPAtE2IMX313P while the remaining 3 groups were administered a combination of pVax + p-tPAtE1tE2IMX313P (Fig. 6.2A). The mice were immunised 4 times with DNA at 3-week intervals (Fig. 6.2B), before receiving a DNA boost (groups 2 & 3; Fig. 6.2A), a tE1tE2 recombinant protein boost (groups 4 & 5; Fig. 6.2A) or a HCV VLP boost (groups 6 & 7; Fig. 6.2A). The inoculations were administered via the intradermal route in the ear pinnae. Each group of animals was vaccinated with a total of 20.65 picomoles DNA per dose per animal and boosted with 50µg of recombinant tE1tE2 proteins or 25µg of HCV VLPs. Blood was collected two days prior to each vaccination and 21 days after the final vaccination when the mice were sacrificed. The spleen was removed, splenocytes prepared for IFN-γ ELISPOT analysis and serum analysed for the presence of anti-E1/E2 antibodies.

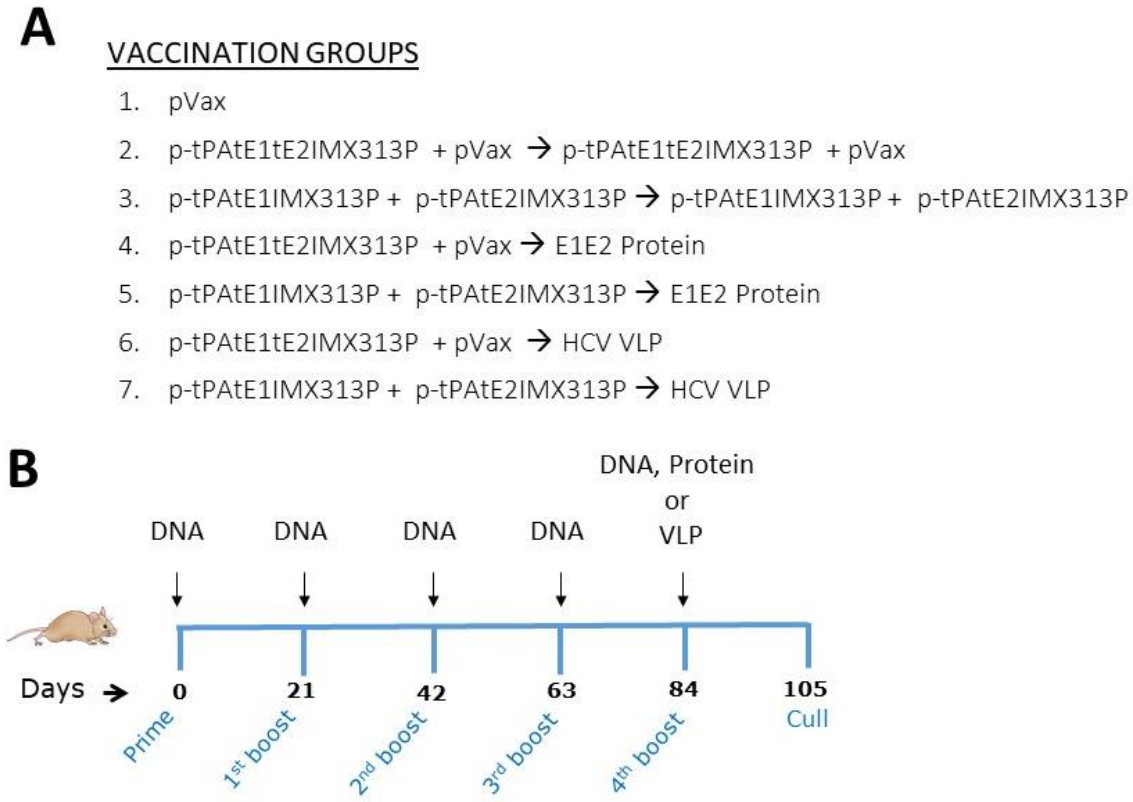


Figure 6.2. Prime/boost vaccination regimens in 6-8 week old female Balb/C mice. Vaccination groups (A) and immunisation schedule of animals (B). Female Balb/C mice (n= 7/group) were inoculated intradermally with 20.65 picomoles of the respective plasmids at three weekly intervals and boosted with DNA, recombinant HCV tE1tE2 protein or HCV VLPs. pVax was included in the vaccine cocktails to ensure that all animals were vaccinated with an equimolar amount of DNA. Blood was collected two days prior to each vaccination and 21 days post vaccination. Spleens were also harvested 21 days post the final vaccination.

6.3.3 Immune responses

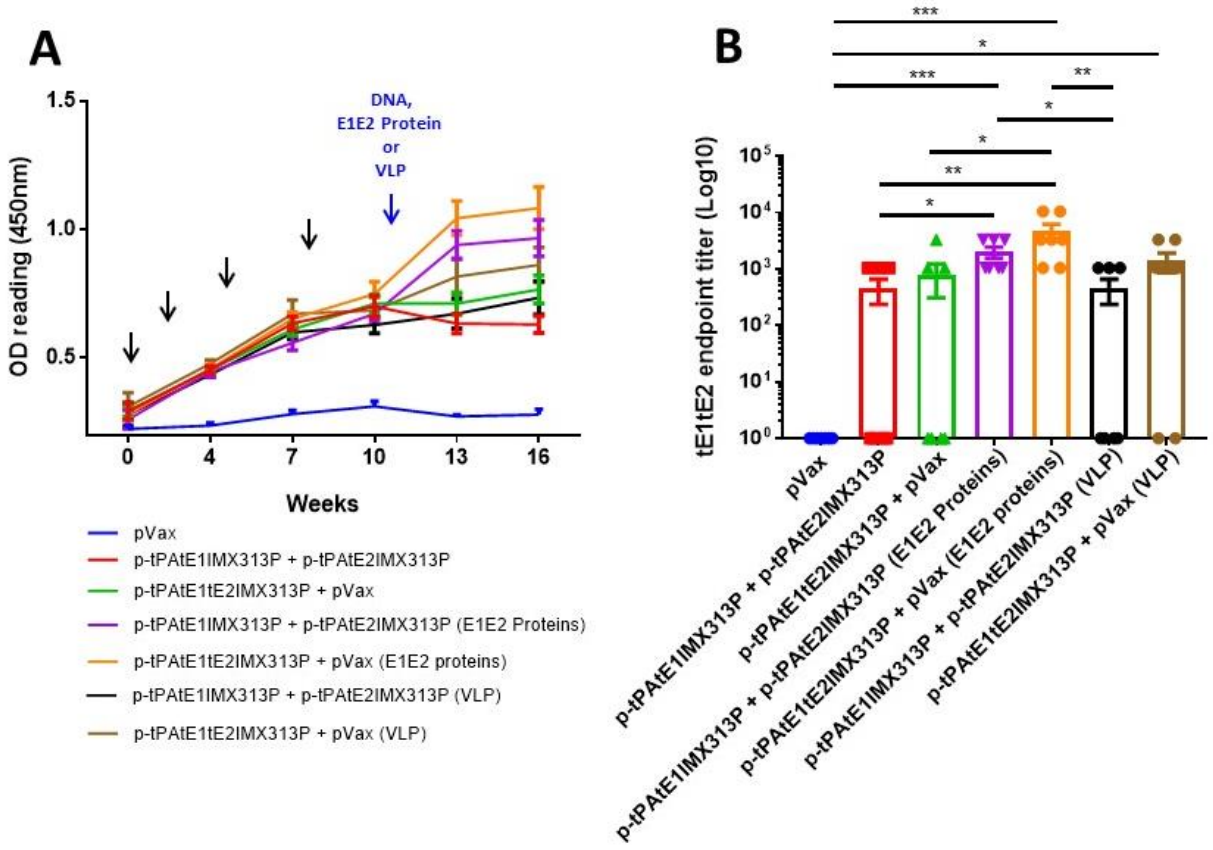
6.3.3.1 Humoral responses

6.3.3.1a Serum antibody responses

To determine the antibody responses, mouse serum samples were collected and analysed for E1E2-specific antibodies by ELISA. As previously observed (chapter 5 section 5.3.4.1), the antibody levels peaked and plateaued following the 3rd booster immunisation in the group receiving DNA prime/DNA boost regimens (Fig. 6.3A). Vaccination with a cocktail of p-tPAtE2IMX313P + pVax and boosting with E1E2 protein resulted in the highest tE1tE2-specific antibody levels, followed by p-tPAtE1IMX313P + p-tPAtE2IMX313P prime/ E1E2 protein boost and p-tPAtE2IMX313P + pVax prime/ HCV VLP boost (Fig 6.3A).

The antibody titers measured 3 weeks post the final vaccination showed that boosting with the tE1tE2 proteins following immunisation with a cocktail of p- tPAtE1tE2IMX313P + pVax resulted in the highest anti-E1E2 antibody response with an average titer of 1/4678, followed

by p-tPAtE1IMX313P + p-tPAtE2IMX313P-vaccinated mice (average titer 1/2004). The DNA prime/E1E2 protein boost vaccination regimens were significantly superior compared to boosting with DNA alone or HCV VLPs. There were no statistical differences in antibody titers against E1E2 between animals vaccinated with p-tPAtE1tE2IMX313P + pVax and boosted with tE1tE2 proteins or HCV VLPs (average titer 1/1386) (Fig. 6.3B). No significant differences were observed in E1E2-specific antibody titers between mice vaccinated with p-tPAtE1tE2IMX313P + pVax or p-tPAtE1IMX313P + p-tPAtE2IMX313P and boosted with the same DNA or HCV VLPs (average titer 1/1386) (Fig. 6.3B). These antibodies were also capable of binding to full length E1E2 as a heterodimer as determined by immunofluorescence (Fig. 6.3C). Collectively, the data indicate that recombinant E1E2 protein provides a superior boost compared to DNA or HCV VLPs and is capable of enhancing HCV E1E2-specific antibody responses in p-tPAtE1tE2IMX313P + pVax or p-tPAtE1IMX313P + p-tPAtE2IMX313P-vaccinated mice.



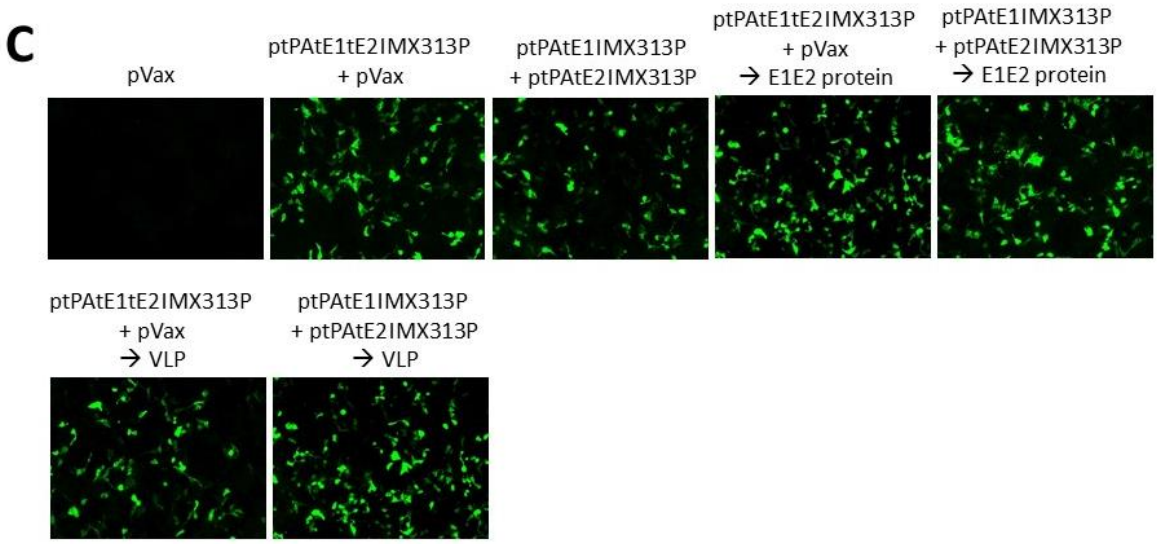


Figure 6.3. Induction of anti-E1 and anti-E2 antibodies following vaccination with DNA prime/DNA boost, DNA prime/E1E2 protein boost or DNA prime/HCV VLP boost regimens. A) Serum antibody response specific for HCV E1E2 proteins. Serum from vaccinated mice was diluted (1/50) and anti-E1E2-specific antibodies were measured by ELISA at the indicated time points. Black arrows indicated when the DNA vaccine was administered, blue arrow indicate when the DNA, protein or VLP boosts were administered. Values represent mean responses in each group (n = 7) ± SEM. B) Three weeks after the final immunisation, serum from individual mice was serially diluted, and endpoint binding titers were calculated. Values for individual mice are shown (n = 7) and bars represent the mean ± SEM. Mann-Whitney non-parametric t-test was performed to assess significant p-values between the vaccinated groups. *$p<0.05$, **$p<0.01$, *** $p < 0.001$. C) Immunofluorescence analysis of the presence of antibodies specific for full-length E1 and E2 proteins in immune sera. HEK293T were transfected with p-CE1E2-PRF(DA) encoding full length E1E2 and pooled sera from mice vaccinated with DNA prime/DNA boost, DNA prime/E1E2 recombinant protein boost or DNA prime/HCV VLP boost regimens were used as the primary antibody at a 1:50 dilution.

6.3.3.1b Serum antibody characterisation

The specificities of the antibody responses to continuous and discontinuous epitopes on E1E2 were mapped by overlapping peptides and competition ELISA. To map antibody responses to continuous epitopes, a library of E1E2 overlapping peptides (18-mers with 11-amino-acid overlaps) was used in an ELISA (Fig. 6.4). There was little difference in reactivity with the E1 continuous epitopes located in the ectodomain in all the immunised groups; sera from animals immunised with the p-tPAtE1tE2IMX313P + pVax DNA prime/ E1E2 protein boost regimen or the DNA prime/ HCV VLP boost regimen showed a slightly higher reactivity compared to

other immunised groups (Fig. 6.4A). There were no differences in reactivity to the E1 peptides from the transmembrane domain across all vaccination groups. However, the sera showed greater reactivity against the E2 glycoprotein than the E1 glycoprotein and reacted with aa 412 to 522 and aa 541 to 713 (Fig.6.4B). All vaccinated groups showed little reactivity toward hypervariable region 1 (HVR1) aa 384 to 411 and E2 TMD region aa 716 to aa 747 but some reactivity against E2 HVR 2 aa 460 to 485 and igVR aa 570 to 580 (Fig. 6.4B).

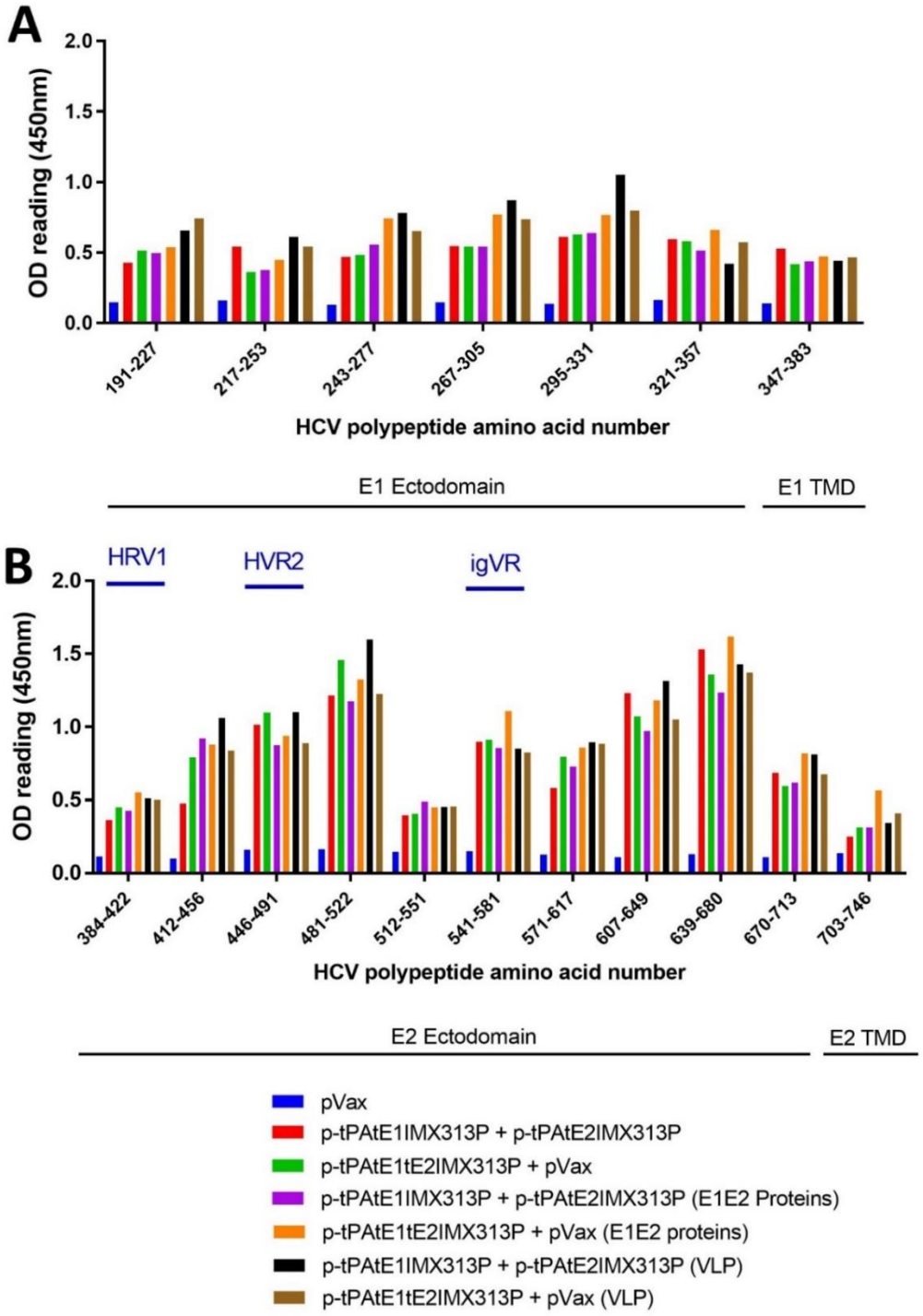


Figure 6.4. Reactivity of sera from DNA prime/DNA boost, DNA prime/E1E2 protein boost or DNA prime/HCV VLP boost-immunized animals to E1E2 peptides. Serum antibody responses specific to a library of overlapping (A) E1 or (B) E2 peptides in ELISA three weeks after the last vaccination. Serum from vaccinated mice was diluted (1/50) and peptides were supplied by BEI Resources. Values represent mean responses in each group (n = 7) + SEM. Mann-Whitney non-parametric t-test was performed to assess significant p-values between the vaccinated groups. **p<0.01. Data shown are representative of those from 3 independent experiments performed in duplicate.

To map antibody specificities to discontinuous epitopes, a panel of human monoclonal antibodies (MAbs), (HC33.1, HCV1, AR3C, 2AI2 and HC84.27– kindly provided by Professor Heidi Drummer), capable of binding to the E2 protein were used in competition ELISA (Fig.6.5). These MAbs target the regions of E2 that form the CD81 receptor binding site to block the interaction between E2 and CD81 [152, 160, 192, 490-492]. Competition ELISA was used to determine if similar epitopes were targeted by vaccine-elicited antibodies. Preliminary titration experiments with the MAbs in E1E2-binding ELISAs were performed and the concentration of the MAbs resulting in 70% maximal binding was used in the competition studies (data not shown). The effects of serum from immunised mice on binding of the MAbs was assessed by first adding mouse serum to E1E2-coated ELISA plates, followed by the addition of the individual MAbs, as described previously [453, 493]. The reduction in absorbance of the monoclonal antibodies was then calculated. Animals vaccinated with p-tPAtE1tE2IMX313P + pVax DNA prime/ DNA boost or p-tPAtE1tE2IMX313P + pVax DNA prime/ E1E2 protein boost showed the greatest reactivity toward the HC33.1 epitope with 16.7% competition and 16.1% competition respectively (Fig. 6.5A), while p-tPAtE1IMX313P + p-tPAtE2IMX313P DNA prime/ E1E2 protein boost showed the highest reactivity (16.2% competition) toward the HCV1 epitope (Fig. 6.5B). All vaccination regimens resulted in similar competition with the AR3C (Fig.6.5C), 2AI2 (Fig. 6.5D) and HC84.27 (Fig. 6.5E) antibodies. DNA prime/ VLP boost-immunised animals showed the lowest competition with all five MAbs (Fig.6.5A-D). Due to the lack of sera the MAb competition studies were only performed using a 1/50 dilution of the serum from the vaccinated mice. Nevertheless, the results show that sera from vaccinated mice recognise E2-neutralising epitopes originally defined by the HC33.1, HCV1, AR3C, 2AI2 and HC84.27 MAbs.

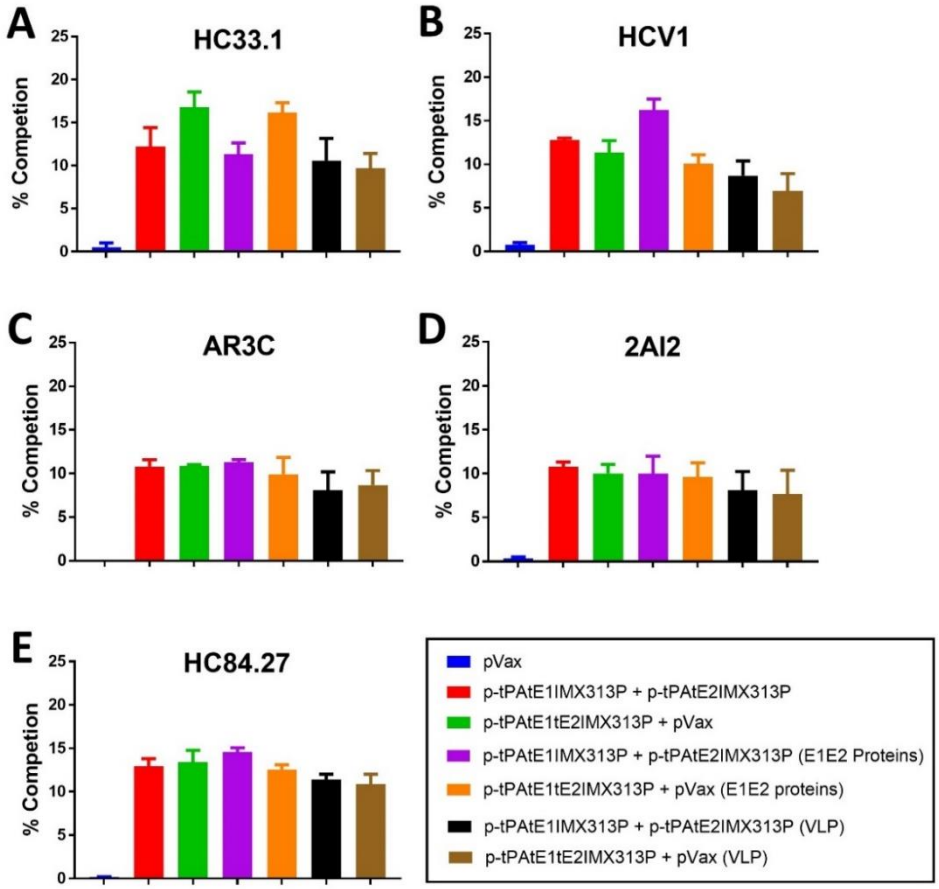


Figure 6.5. Competition ELISA of antibodies from vaccinated mice with human MAbs recognising five specific epitopes. Pooled serum from vaccinated mice was diluted (1/50) and first incubated for 1 hour on E1E2-coated plates, followed by the addition of the indicated MAb. Monoclonal antibodies were added at a concentration resulting in 70% maximal binding to E1E2, as determined in prior titration experiments. Binding of the MAbs was detected with anti-human HRP-conjugated secondary antibodies. Percentage reduction in absorbance of the monoclonal antibodies was calculated and plotted as % competition. The data are reported as the mean + SEM of at least 2 experiments performed in duplicate.

6.3.3.2 Serum HCV neutralising activity

To assess the neutralising ability of the antibodies generated by the different vaccination regimens, the ability of the sera to inhibit binding of HCV VLPs to Huh7 cells was investigated. This component of the study was performed by Professor Joseph Torresi. Different dilutions of serum (1:5, 1:10 and 1:25) were incubated with fluorescent-labelled HCV VLPs, and the binding of the VLPs to Huh7 cells was monitored as stated in chapter 2 - section 2.2.6.4. Incubation of the HCV VLPs with PBS (VLP only) or normal mouse serum (Sigma) resulted in minimal inhibition of HCV VLP binding to hepatocytes (Fig. 6.6B-middle panel). The addition of anti-CD81 antibodies (Fig.6.6C), on the other hand, inhibited VLP binding to Huh7

cells by >90% confirming that these VLPs use this molecule to facilitate binding to target cells, in a similar manner to HCV [68, 77, 126, 243, 494, 495].

Dose-dependent inhibition of binding of the HCV VLPs by serum from the vaccinated mice was observed across all vaccination groups (Fig. 6.7A). Although most of the samples showed some non-specific inhibition at a 1:5 dilution, at a 1:25 dilution which was considered to represent specific inhibition, neutralisation of binding of HCV VLPs to Huh7 cells was highest by sera obtained from mice immunised with a DNA prime/DNA boost or a DNA prime/tE1tE2 boost (~71% for p-tPAtE1IMX313P + p-tPAtE2IMX313P prime/ DNA boost, ~64% for p-tPAtE1tE2IMX313P + pVax prime/ DNA boost, 60% for p-tPAtE1IMX313P + p-tPAtE2IMX313P prime/ E1E2 protein boost or 64% for p-tPAtE1tE2IMX313P + pVax prime/ E1E2 protein boost), compared to sera obtained from mice boosted with HCV VLPs (~41% for p-tPAtE1IMX313P + p-tPAtE2IMX313P prime/HCV VLP boost or ~30% for p-tPAtE1tE2IMX313P + pVax prime/ HCV VLP boost; Fig. 6.7B). Neutralisation of binding following boosting with HCV VLPs was surprisingly low (~27%) and similar to that of sera obtained from the pVax control group (Fig. 6.7B). The representative graphs used to calculate percentage inhibition are also shown (Fig. 6.7). These results show that antibodies generated against HCV genotype 1 E1/E2 proteins could neutralise the binding of VLPs to target cells, and that homologous DNA prime-boost vaccination with p-tPAtE1IMX313P + p-tPAtE2IMX313P resulted in the highest neutralisation activity.

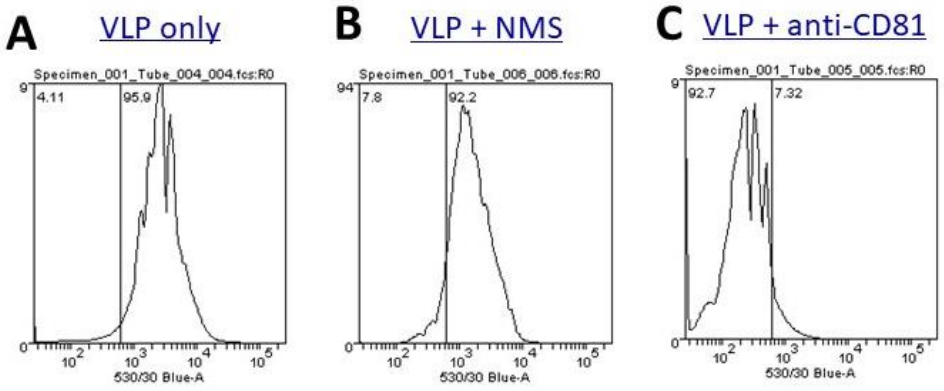


Figure 6.6. Inhibition of binding of HCV-LPs to Huh7cells by mice sera, as determined by flow cytometry. Huh7 cells were incubated with A) fluorescent-labelled VLPs in the presence of B) normal mouse serum (NMS; Sigma) or C) anti-CD81 antibody. Cells were then washed and analysed for fluorescence by flow cytometry. This experiment was performed by Professor Joseph Torresi.

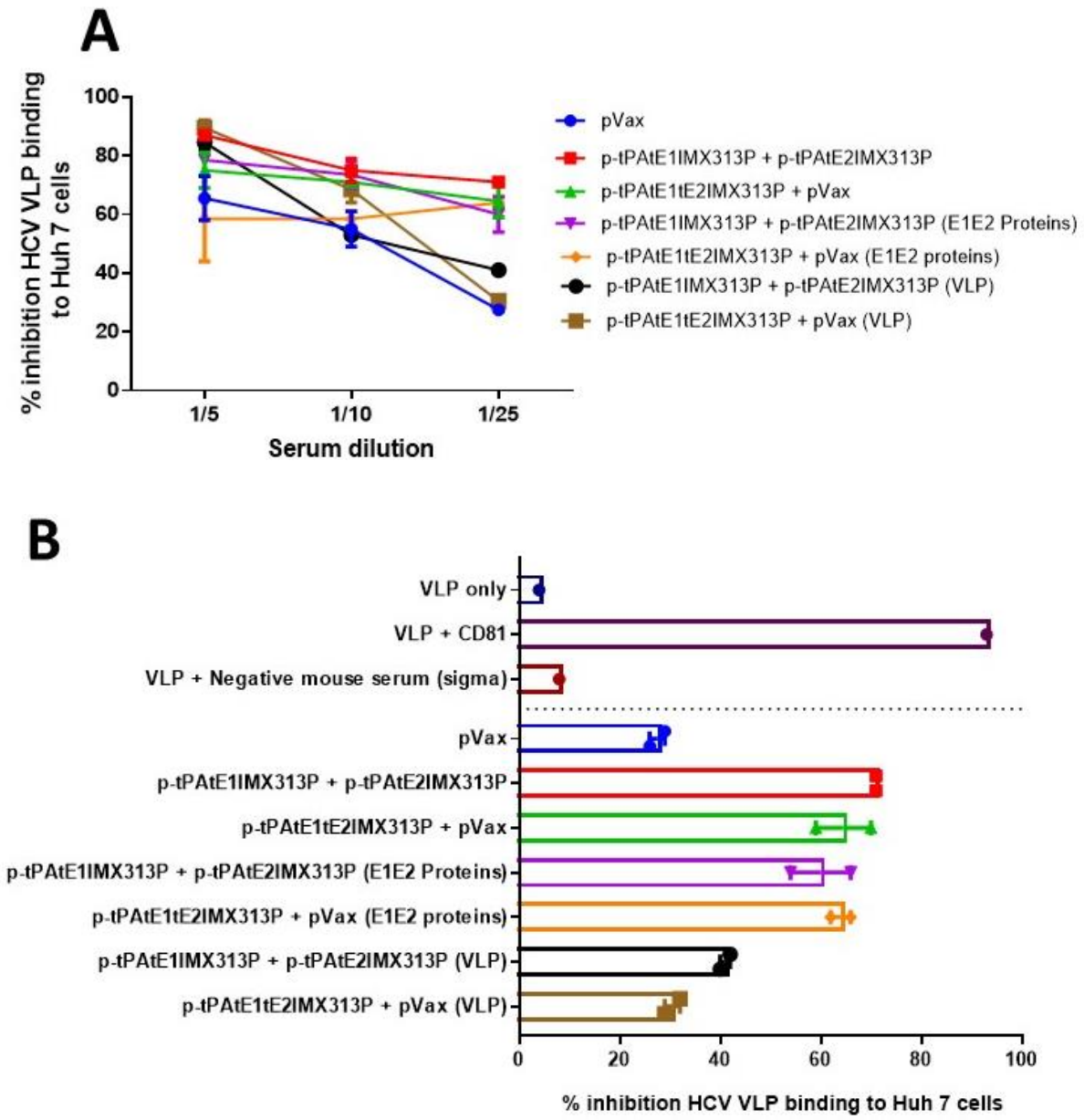


Figure 6.7. Neutralisation of VLP binding by sera from vaccinated animals after the final boost immunisation. Neutralisation of VLP binding was determined by pre-incubating fluorescent-labelled VLPs with 1:5, 1:10 or 1:25 dilution of immune sera from mice inoculated with the various prime-boost vaccination regimens. Supernatants were clarified by centrifugation and incubated with Huh7 cells. Cells were then harvested, and cellular fluorescence levels analysed by flow cytometry. A) Fluorescent-labelled HCV VLPs of genotype 1b were incubated with decreasing concentration (1:5, 1:10, and 1:25) of serum from vaccinated mice after the final immunisation. Y-axis depicts the percentage inhibition of binding of HCV-LPs to Huh7 cells; X-axis represents the different dilutions of serum. B) All bar graphs represent the percentage reduction in VLP entry following incubation with mouse sera at 1:25 dilution. This experiment was performed by Professor Joseph Torresi.

In addition to their neutralising properties, antibodies can mediate host effector functions and facilitate the removal of a pathogen from a host. The Fc portion of immunoglobulin G2a (IgG2a) antibodies in particular, can interact with complement components [496] and Fc receptors [497-499]. This interaction can lead to cell death through complement fixation, stimulation of antibody-dependent cell-mediated cytotoxicity (ADCC) [500], and phagocytosis [501]. E1E2-specific total IgG and IgG2a isotyping demonstrated that the anti-E1E2 humoral response was predominantly comprised of IgG subclasses other than IgG2a ($p < 0.01$) (Fig. 6.8), suggesting that these antibodies are unlikely to show ADCC activity or to be involved in complement activation. Further studies are necessary to confirm these observations.

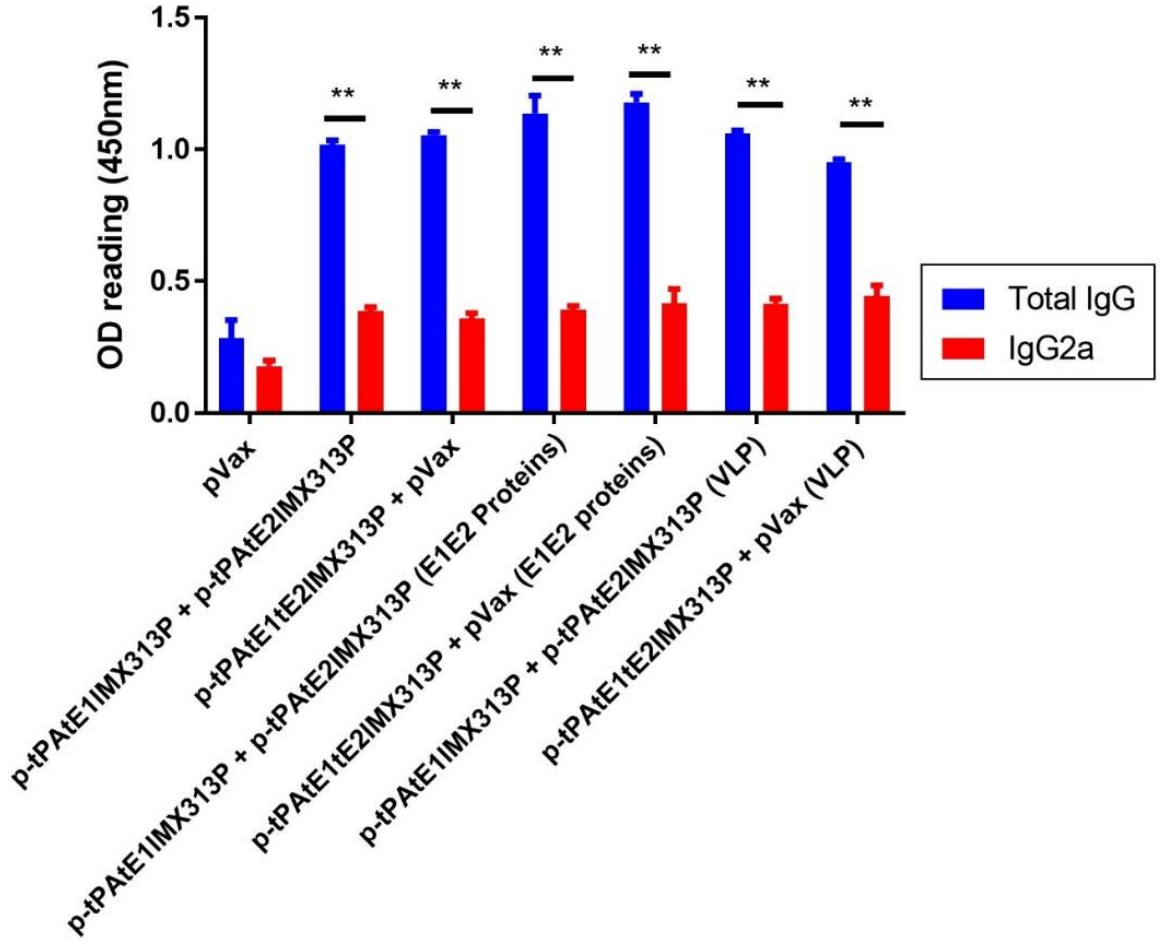
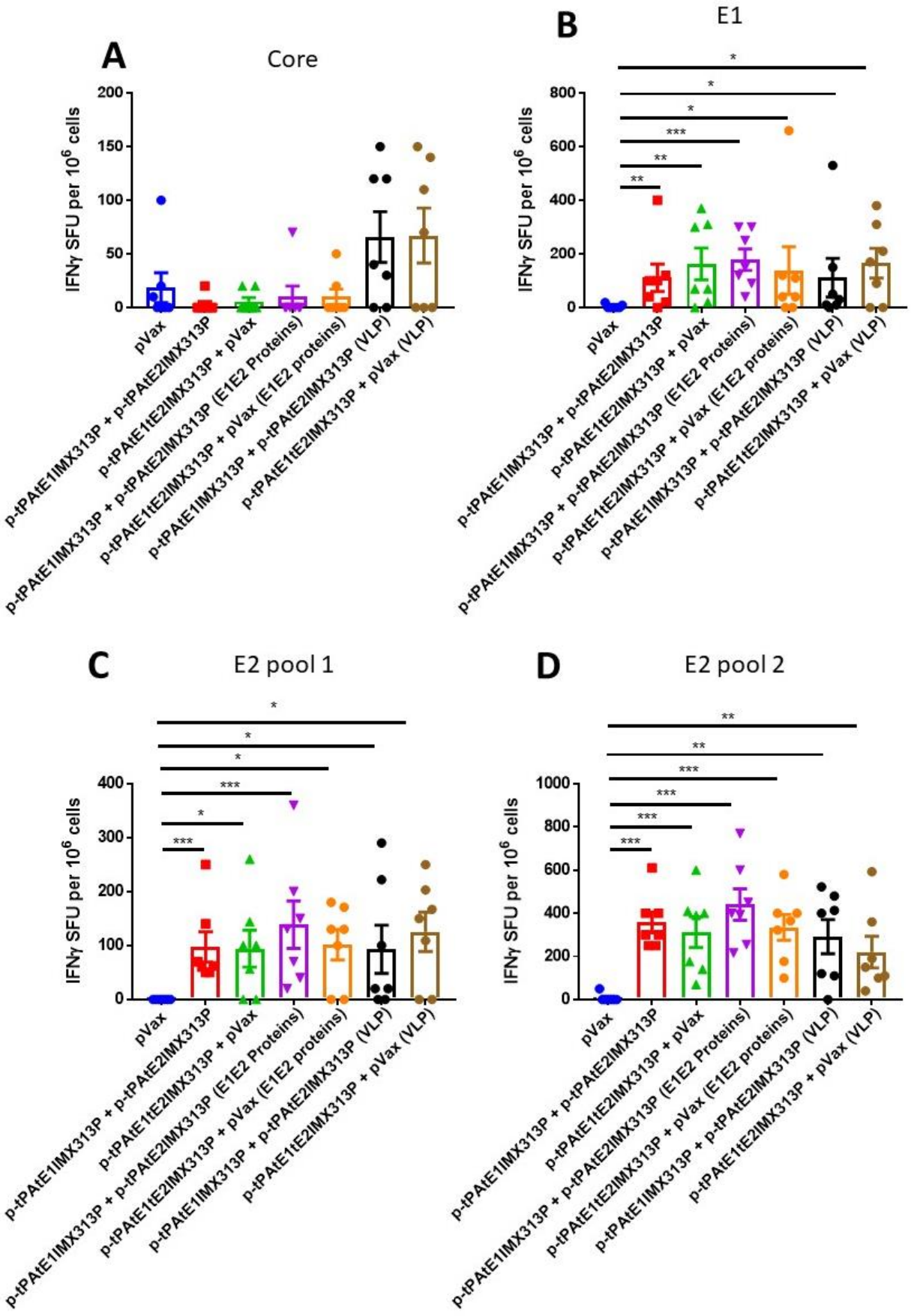


Figure 6.8. Levels of anti-E1E2 IgG and IgG2a following vaccination with DNA prime/DNA boost, DNA prime/E1E2 protein boost or DNA prime/HCV VLP boost regimens. The magnitude of E1E2-specific total IgG or IgG2a isotype of immune sera from immunised mice was determined by ELISA, using secondary antibody recognising (blue bars) all IgG subclasses (total IgG) or (red bars) IgG2a isotype-specific secondary antibody. Values represent mean responses in each group (n = 7) + SEM. Mann-Whitney non-parametric t-test was performed and significant p-values between groups are shown. **p<0.01.

6.3.3.3 Cell-mediated immune responses

To compare CMI elicited by the different regimens, an IFNγ ELISPOT assay was performed using peptide pools representing HCV core, E1 or E2 proteins for re-stimulation (Fig. 6.9). As shown in figure 6.9A, mice boosted with HCV VLPs showed a higher frequency of IFN-γ-secreting T-cells following stimulation with core peptides, with ~65 SFU/10^6 cells for the p-tPAtE1IMX313 + p-tPAtE2IMX313 prime/ HCV VLP boost regimen and ~67 SFU/10^6 cells for the p-tPAtE1tE2IMX313 + pVax prime/ HCV VLP boost regimen (Fig. 6.9A) compared with mice that received the DNA prime/ DNA boost regimens or DNA prime/ E1E2 protein boost regimens. However, these differences were not statistically significant. As expected, since neither the DNA nor the E1E2 recombinant proteins encoded/expressed the core protein, the core-specific responses induced by the DNA prime/DNA boost or DNA prime/protein boost regimens were low, essentially baseline (Fig. 6.9A). In contrast, the IFNγ responses were high in mice from each vaccination regimen following stimulation with E1 (Fig. 6.9B) or E2 peptides (Fig 6.9 C, D & E) but no significant differences were observed between the different vaccination regimens. In an attempt to compare the responses elicited by the mice in this experiment with published data, a composite of the total responses to E2 pool 1 and pool 2 was generated (Fig 6.9E). This showed that these responses compared well with those generated in other studies following vaccination with viral vectors (~140 to ~1600 SFU/10^6 cells for E1 and ~120 to ~800 SFU/10^6 cells for E2 [438, 443, 445]), DNA (~480 SFU/10^6 cells for E1E2 [424]), or VLPs (~190 SFU/10^6 cells for coreE1E2 [421] or ~480 SFU/10^6 cells for E1E2 [424]). As expected, the control mice vaccinated with pVax failed to generate any IFNγ-positive cells against each of the peptide pools (Fig. 6.9A, B, C, D & E).


A
Core
B
E1
C
E2 pool 1
D
E2 pool 2
IFNγ SFU per 10^6 cells
pVax
p-tPAtE1IMX313P + p-tPAtE2IMX313P
p-tPAtE1IMX313P + p-tPAtE2IMX313P + pVax
p-tPAtE1tE2IMX313P (E1E2 Proteins)
p-tPAtE1IMX313P + p-tPAtE2IMX313P (E1E2 proteins)
p-tPAtE1IMX313P + pVax (E1E2 proteins)
p-tPAtE1tE2IMX313P (VLP)
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p-tPAtE1tE2IMX313P + pVax (VLP)

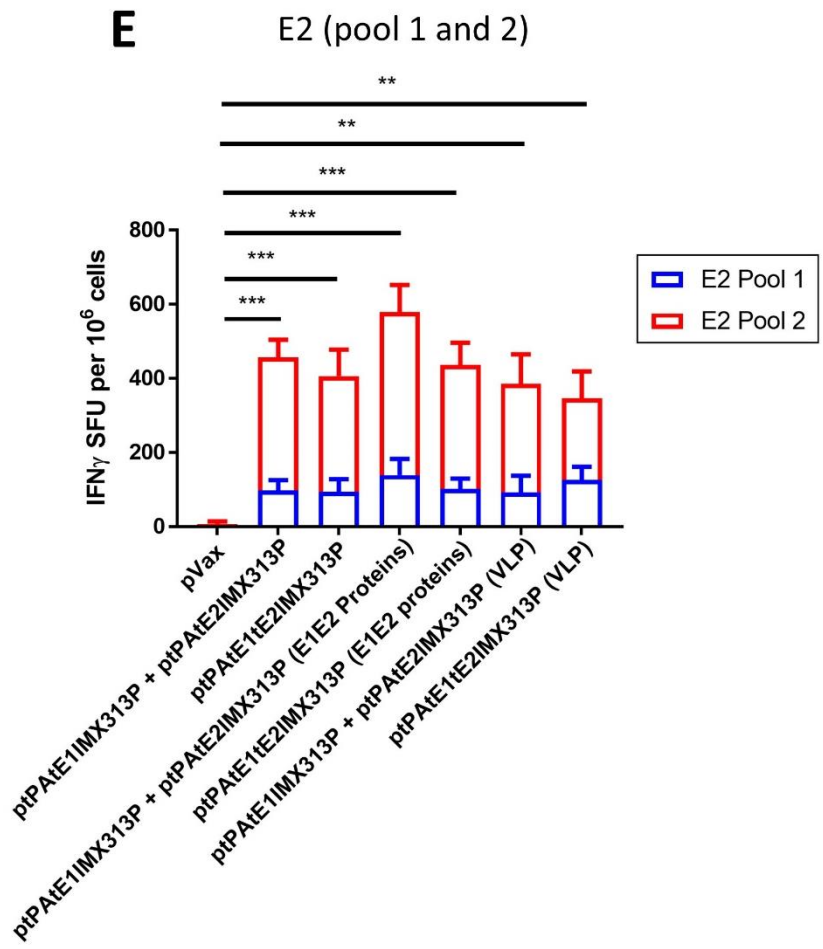

Figure 6.9. HCV-specific cellular immune responses detected in mice immunised with DNA prime/DNA boost, DNA prime/E1E2 protein boost or DNA prime/HCV VLP boost regimens. CMI detected by ELISpot. ELISpot assay of splenocytes after stimulation with HCV peptide pools representing (A) core, (B) E1 and (C & D) E2. (E) represents the combined responses after stimulation with E2 peptide pools 1 and 2. The data are expressed as spot forming units (SFU) per 10^6 cells to different peptide pools and presented as the mean ±SEM for seven mice per group. The number of SFU in unstimulated splenocytes was subtracted from the number in peptide-stimulated cells to generate the net HCV response. Mann-Whitney non-parametric t-test was performed and significant p-values between the vaccinated groups are shown. *$p<0.05$, **$p<0.01$. Note that the Y-axes may differ.

6.4 Discussion

DNA vaccination is able to induce humoral and cellular immunity against various infectious agents. Despite this, poor immunogenicity in large animals remains one of the major limitations of DNA immunisation [502]. Multiple immunisations are commonly required to achieve a protective and sustained immune response. The DNA prime-protein boost strategy, in which the immune response is primed with a DNA vaccine and subsequently boosted with a protein, VLP or vector expressing the antigen, constitutes a promising approach to improve the efficiency of DNA immunisation [503-505].

In this chapter, mice were immunised on four occasions with DNA vaccine constructs encoding tE1/tE2-IMX313P before boosting with DNA, E1E2 protein or HCV VLPs, and the efficacy of these vaccination strategies to elicit antibody responses and cell-mediated immunity against the HCV structural proteins was determined. It was observed that the DNA prime/DNA boost, DNA prime/E1E2 protein boost, and DNA prime/HCV VLP boost vaccination methods induced quite different immune responses. Among the three immunisation strategies, boosting with one dose of recombinant E1E2 protein induced the highest antibody levels as determined by ELISA. No significant differences were observed between boosting with DNA or HCV VLPs.

The E2 HVR1 (aa 384 to 410) has been suggested to be an immunodominant region involved in viral escape of antibodies in infected chimpanzees and humans [140, 506-508]. There was little reactivity toward this region in the mouse sera, similar to the results of previous studies [453, 509, 510]. This suggests that the E2 HVR1 may be less immunogenic than considered previously and it is thought to be involved in shielding more conserved E2 epitopes [168, 170]. Alternatively, the E2 HRV1 region might have not been efficiently exposed *in vivo* to prime B cell resulting in low levels of HRV-specific antibodies. Additionally, NAb induced in mice have been suggested to recognise different epitopes to those induced in humans [511]. The mouse sera competed with MAbs HC33.1 [160], HCV1 [192, 490] AR3C [491], 2AI2 and HC84.27 [492] in binding to E1E2, suggesting that antibodies to the discontinuous antigenic regions were elicited.

The ability of these antibodies to inhibit the binding of HCV VLPs to Huh7 cells was assessed and sera from mice vaccinated with the DNA prime/DNA boost or DNA prime/E1E2 protein boost regimens were able to prevent VLPs binding to Huh7 cells. This suggests that these antibodies are likely to possess neutralising properties. In contrast and somewhat surprisingly, the prime-boost protocol, in which the DNA vaccine was followed by a boost with HCV VLPs resulted in minimal inhibition of binding of HCV VLPs to Huh7 cells, suggesting that these antibodies were either of low affinity or bound to sites close to but distinct from the neutralising epitopes. These results contradict previous findings which demonstrated that immunisation with HCV VLPs induced HCV-specific NAbs, since the envelope proteins are presented on the surface of VLPs in their correct conformation during immunisation [420, 421]. An explanation for these disparities could be that the antigen dose in the E1E2 protein boost was higher than that expressed on the surface of VLPs resulting in higher immune responses following boosting. Antibody isotyping revealed that the anti-E1E2 antibody response was predominantly comprised of other IgG subclasses but not IgG2a which is a hallmark of a dominant Th1 lymphocyte response, important for mediating complement and ADCC activities [496-501].

However, the anti-E1E2 antibody subclass profiles were not comprehensively defined given that the assay used in this chapter focussed on isotyping E1E2-specific IgG2a antibodies. Future studies should further define the anti-E1E2 IgG subclasses as well as assess their involvement in complement activation and ADCC. The ability of the mouse sera to prevent HCV VLPs binding to Huh-7 cells should be assessed throughout the vaccination schedule to determine whether the limited inhibition of binding was a direct result of the HCV VLPs boost, to eliminate the unlikely possibility that this group of animals were immunocompromised or that the vaccination regimen was somehow inadequate. The presence of NAb acting at the post-binding stages should also be assessed using the HCVcc and HCVpp model systems. It would also be useful to test the potential of the antibodies generated with all three regimens to neutralise HCV particles from other genotypes.

Spontaneous resolution of HCV is also associated with an early and durable cytotoxic T-lymphocyte response with IFN-γ^+ production, and the presence of weak and/or functionally impaired $CD8^+$ T cells is a characteristic feature of persistent infection [26, 93, 512-514]. To examine the production of IFNγ from T cells following vaccination with the DNA constructs, IFNγ ELISPOT analysis against HCV peptides was conducted. There were no significant differences in IFNγ responses following stimulation with E1 or E2 peptides in all vaccination regimens. The number of SFUs detected in the E1/E2-specific ELISPOT reported in this chapter compared well to those reported in other studies using viral vectors [438, 443, 445], DNA [424] or VLPs [421, 424].

Overall, the results indicate that the DNA prime/E1E2 protein regimens described in this chapter elicited a stronger antibody response than DNA prime/DNA boost regimens or DNA prime/HCV VLP protein regimens, whereas the serum neutralising activities were slightly higher for the DNA prime/DNA boost regimens than the DNA prime/E1E2 protein regimens. No significant differences in CMI responses were observed between all three vaccination regimens. Recognised difficulties in HCV neutralisation assays [490, 515, 516] and the inability of current *in vitro* assays to identify effector mechanisms which correlate with protection, suggest that in *vivo* vaccination followed by challenge experiments are paramount to assess the efficacy of potential HCV vaccines.

6.5 Conclusion

In this chapter, mice vaccinated with p-tPAtE1IMX313P + p-tPAtE1IMX313P or p-tPAtE1tE2IMX313P + pVax were boosted with DNA, E1E2 protein or HCV-VLPs in an attempt to further improve the immunogenicity of the tE1/tE2-IMX313P DNA constructs. All vaccination regimens induced high levels of HCV-specific cell mediated and antibody

responses. Most notably, the induced antibodies possessed neutralising properties. Boosting with E1E2 proteins improved the overall antibody responses as determined by ELISA, compared to boosting with HCV-VLPs or plasmid DNA, however this did not improve the neutralising potency of these antibodies or CMI responses. Overall, DNA prime/DNA boost regimens and DNA prime/E1E2 protein boost which induced humoral and cellular immunological responses against E1E2 represent a useful vaccine that merits further development.

Chapter 7. General Discussion

An effective vaccine is the best long-term and cost-effective solution to combat HCV globally. Studies in humans and non-human primates have provided insight into the possible correlates of protection, however the actual correlates remain poorly understood. It is generally accepted that an effective HCV vaccines should induce NAbs targeting envelope proteins as a minimum and elicit CMI against the non-structural proteins.

This thesis reports the efficacy of vaccination strategies mainly aimed at eliciting humoral responses to HCV envelope proteins. The lytic VLP vaccine strategy described in chapter 3 highlighted that p-CE1E2-PRF could induce HCV-specific immunity although the responses were lower than those reported in the literature. The thesis also demonstrated that vaccination using DNA encoding HCV envelope proteins fused to the oligomerisation domains IMX313 (chapter 4) or IMX313P (chapter 5) significantly increased the HCV-specific humoral and cell-mediated immune responses in vaccinated mice. Vaccination with DNA encoding HCV envelope proteins fused to IMX313P induced the highest humoral and cell mediated immunity and these DNA constructs were used in various prime-boost vaccination regimens in an attempt to increase the vaccine's immunogenicity. Boosting with recombinant HCV proteins increased the E1E2-specific antibody titers but failed to increase the neutralisation activity of these antibodies compared to boosting with DNA. Thus, data generated in this PhD thesis provide insights for the further development of a HCV prophylactic vaccine.

7.1 Improving HCV-VLP DNA vaccine immunogenicity using perforin as adjuvant

Recombinant DNA vaccines offer several advantages over traditional vaccine strategies and can induce both humoral and cell mediated immune responses. However, no DNA vaccines have been licensed for use in humans and this has been attributed largely to the lack of immunogenicity. Nevertheless, recent studies have demonstrated that DNA vaccines against human papillomavirus (HPV) were well tolerated and elicited antigen-specific immunity that controlled HPV viral load and inhibited the progression of tumour lesions and cervical cancer in humans [517, 518]. These studies demonstrated that DNA vaccines can be effective in humans. VLPs, on the other hand, have provided highly encouraging results in clinical trials as effective immunogens due to their highly organised particulate structure [489, 519-521].

The addition of perforin as a genetically-encoded adjuvant within a DNA vaccine has been shown to increase the immune response to HCV-NS3 [418] and NS3,4 & 5 [414] and HIV gag [522]. The hypothesis underlying the work described in chapter 3 was that PRF-induced necrotic cell death would not only enhance the HCV-specific-immune responses via the induction of DAMPs, recognised natural adjuvants, but would also result in release of VLPs formed

intracellularly. It was considered that this strategy would combine the ease of production of DNA vaccines with the immuno-stimulatory properties of VLP vaccines. After administration of p-CE1E2-PRF or p-CE1E2-PRF(DA), mice developed E1E2-specific antibody and cell mediated immune responses. Unfortunately, these responses were weaker compared to those reported in the literature [146, 420, 437, 438] and therefore unlikely to be effective against HCV despite multiple administration of the vaccine. IFNγ secretion is a marker of a Th1 type antigen-specific T cell immune response that plays a key role in fighting virus infection, and multi-functional T cells have been shown to be associated with virus control in HCV infection [432]. Although vaccination with p-CE1E2-PRF induced a HCV-specific T cell-mediated immune response, as determined by IFNγ secretion, particularly to the E2 peptides, these results were weaker when considered in the context of other studies [420, 441, 442].

Other researchers have focused on generating VLP-based DNA constructs, known as plasmo-retroVLPs. These constructs have been engineered to generate *in situ* retroVLPs pseudotyped with E1 and E2 proteins after *in vivo* delivery of the DNA [483, 523, 524]. Unlike the results generated in chapter 3, administration of the plasmo-retroVLPs resulted in the induction of significantly higher antigen-specific responses and antiviral immune protection than standard DNA plasmids [523, 524]. These plasmo-retroVLPs DNA constructs were also shown, in heterologous prime-boost immunisation strategies, to be superior for boosting CMI and antibody responses in primed animals than control plasmids unable to form VLPs [483, 523]. Furthermore, vaccination with a cocktail of plasmo-retroVLPs pseudotyped with E1E2 from five HCV genotypes (1a, 1b, 2b, 3a, 4c and 5) and/or displaying NS3 antigen in capsid proteins resulted in immune responses against all five HCV genotypes [424]. Nonetheless, despite these promising results, even the plasmo-retroVLPs strategy required booster immunisations with purified HCV VLPs for maximum activity.

The poor responses observed using the CE1E2-PRF strategy could be attributed to various factors, including an insufficient uptake of the DNA vaccine *in vivo* by cells in the intradermal environment or an insufficient or partial release of the VLPs *in vivo* following PRF-induced cell death, leading to insufficient priming of the HCV-specific response. The delivery route and/or method of DNA-based vaccines is also a critical factor in determining vaccination outcome. The immunogenicity of the plasmo-retroVLPs DNA vaccine was shown to be influenced by the route of DNA injection and was increased by co-delivery of plasmid DNA encoding cytokines cytokine genes or CpG sequences [424, 523]. Administration of HCV DNA vaccines using *in vivo* electroporation has also been shown to enhance the vaccine immunogenicity by increased local DNA uptake, protein expression, inflammation, and infiltration of T cells [277, 321, 481]. Nonetheless, despite the ability of electroporation to

improve DNA delivery and immunogenicity of the encoded antigen, it was not used in this thesis as it is associated with considerable discomfort and tissue damage which might limit its long-term future in vaccination. Therefore, attempts should be directed towards optimisation of the delivery methods of DNA-based HCV VLP vaccines and the addition or co-delivery of other novel genetic adjuvants for maximum immune induction. The effect of the p-CE1E2-PRF vaccine constructs in a prime/boost strategy should also be investigated, for example using a rAd5-CE1E2-based HCV vaccine, HCV VLPs [420] or indeed the recombinant E1E2 proteins used in chapter 6, as this may further enhance HCV-specific immune response to levels similar to those reported in the literature.

Although the CE1E2-PRF induced weak HCV-specific antibody responses, with further improvements to the vaccine composition and delivery methods, this strategy could still be used to generate immune responses against HCV.

7.2 Increased DNA vaccine efficacy resulting from E1/E2 heptamer expression

HCV envelope proteins are the target of NAbs because they are exposed on the surface of the virion and represent the most immunologically significant HCV antigens [146, 420, 443-445]. Consequently, these proteins were the main focus of the vaccine strategies described in chapters 4 and 5 of this thesis. Previously, it has been shown that cell surface-expressed E1 and E2 glycoproteins are more effective immunogens than the wild-type antigens [509, 525]. Secreted E1E2 proteins have also been shown to be significantly more immunogenic with a greater breath of reactivity than wild-type E1E2 [453]. Antigen oligomerisation has also been shown to improve vaccine immunogenicity [461-463]. The work presented in chapters 4 and 5 aimed at developing a DNA vaccine encoding E1 and E2 incorporated into heptamers by fusion with the oligomerisation domain of a chimeric C4 binding protein IMX313 or IMX313P. The immunogenicity of DNA vaccines encoding E1 and/or E2 proteins as separate immunogens or as a single E1E2 polyprotein when fused to IMX313 (chapter 4) or IMX313P (chapter 5) was assessed in mice.

All groups immunised with the HCV DNA vaccines encoding E1E2 heptamers elicited significant levels of anti-E1E2 antibodies comparable to those reported in the literature [420, 445, 472]. This may be the result of increased capture and processing of the heptamers by both local and distal antigen presenting cells, thus increasing the immunogenicity of the E1E2 antigens [461-463, 509, 510, 526]. Moreover, these antibodies were also able to recognise E1E2 proteins in their native heterodimer form in an immunofluorescence assay, on cells transfected with a DNA construct encoding the full-length coreE1E2 polyprotein [447, 448, 527]. However, the anti-E1-specific antibody responses were limited, in agreement with the poor

immunogenicity of E1 observed in previous studies [146, 453, 528]. In addition to strong humoral responses, robust CMI responses have been proposed to be essential for clearance of HCV during acute infections in humans and chimpanzees [26, 199, 316, 473]. Fusion of E1/E2 to IMX313P resulted in superior E1/E2-specific antibody and CMI responses compared to fusion of the same proteins to IMX313. This suggested that, in addition to oligomerisation, other factors such as increased half-life, improved antigen uptake and/or prolonged antigen processing could be involved in the enhancement of the E1/E2-specfic responses. X-ray crystal structure studies of the heptamers could provide more information of the mode of action of the IMX313 and IMX313P domains and improve our understanding of how these domains affect the immunogenicity of the fused HCV antigens.

Other HCV vaccine strategies to elicit neutralising antibodies have focused on incorporating highly antigenic conserved epitopes in the vaccine formulation, rather than WT HCV sequences. This was considered to allow the induction of broadly reactive NAbs while eliminating deleterious sequences that may act as immune decoys and/or contribute to isolate-specific structural variations. A recombinant E2-based prophylactic vaccine, was recently shown to generate potent cross-NAbs antibodies in guinea pigs [529]. This recombinant E2 vaccine was generated by removing the variable regions HVR1, HVR2 and igVR from the E2 receptor-binding domain (E2 Δ123) [529, 530]. This strategy generated high-molecular weight forms of E2 Δ123 capable of eliciting distinct antibody specificities with potent and broad neutralizing activity against all seven HCV genotypes [529]. However, the Δ123 protein preparation is predominantly comprised of monomers, together with a range of oligomers. These oligomeric species exhibited distinct antigenic characteristics and immunogenic potential with the high molecular weight species generating the highest NAbs titers. Despite these promising results scaling up the manufacturing process may present a limiting factor for the development of HCV vaccines using this strategy.

The novel E1/E2-IMX313P DNA vaccine described in this thesis is an attractive candidate for a HCV vaccine that could be readily scaled up. A recombinant HCV E1E2 glycoprotein (genotype 1a) vaccine was shown previously to reduce the carrier rate in immunised chimpanzees and generate sterilising immunity in some individuals in response to homologous and heterologous 1a virus challenge [283, 531, 532]. Furthermore, other E1E2 candidate vaccines demonstrated broad NAb responses in vaccinated human volunteers [282, 493, 533, 534]. Soluble forms of E2 can be produced from mammalian and insect cells [76, 178, 470, 535], and represent the focus of efforts in the rational design of a universal B cell vaccine against HCV [536, 537]. However, it is important to note that cross-neutralising epitopes that contain E1 and E2 residues are not represented in soluble E2 proteins [142]. Furthermore,

recombinant gt1b E1, but not recombinant E2, provided protection to immunised chimpanzees from homologous genotype 1b viral challenge [538]. Finally, developing an E1E2-specific NAb response may be a perquisite to mount a successful CMI response for viral clearance [539, 540]. For these reasons, a DNA vaccine encoding E1/E2-IM313P remains a prime candidate for HCV vaccine development.

Collectively, the data reported in this thesis indicate that fusing E1/E2 to IMX313P resulted in secretion of the oligomerised protein and significantly enhanced the immunogenicity of the E1E2-based HCV DNA vaccine, to a level similar to that of other HCV vaccine candidates in preclinical animal studies [26, 199, 316, 473] [424, 443, 445]. These results support the inclusion of IMX313P domain as a molecular adjuvant in a DNA vaccine as an attractive strategy for developing an effective E1E2-based HCV DNA vaccine. Unlike other vaccine strategies such as subunit or VLP vaccines, the p-tE1/tE2-IMX313P vaccine construct, being a DNA vaccine, will be stable and cheaper to manufacture on a large scale as it does not require protein or particle purification.

7.3 Improving DNA vaccination using a prime/boost strategy.

Early vaccination studies in chimpanzees using E1E2 glycoproteins expressed by HeLa cells infected with a recombinant vaccinia vector [283, 541] or with E1E2 expressed in Chinese hamster ovary cell lines [506, 542] induced NAbs with various neutralisation efficacies in different animal models [526, 532]. Vaccine candidates using E1E2 glycoproteins have been found to induce modest NAb responses in human vaccine trials [282, 533, 543], although the NAb titer required to protect against infection with homologous HCV is currently not known.

DNA vaccination provides a convenient platform that can be used to induce humoral and cellular immunity to HCV glycoproteins expressed endogenously. Nevertheless poor immunogenicity in large animals remains one of the major limitation of DNA immunisation [502]. A DNA prime-protein boost strategy is a promising approach to improve the efficiency of DNA immunisation [503-505]. A series of DNA constructs encoding secreted, truncated E1/E2 fused to IMX313P were shown to elicit superior responses as described in chapter 5, while the work described in chapter 6 assessed the ability of HCV VLPs or a soluble form of E1E2 proteins to increase the E1E2-specific responses elicited by DNA vaccination encoding E1/E2-IMX313P fusion proteins. The immunogenicity of these vaccines was compared against homologous DNA prime/DNA boost vaccination. The data showed that all three vaccination regimens induced high levels of E1E2-specific cell mediated and antibody responses. Among the three immunisation strategies, boosting with recombinant E1E2 protein induced the highest

IgG levels while there were no significant differences after boosting with DNA or HCV VLPs. Furthermore, mice vaccinated with these different regimens developed NAbs to HCV VLPs.

To date, virtually all successful viral vaccines have been based on the induction of NAbs, usually via the targeting of viral envelope proteins [544]. For HCV, complete neutralisation of incoming virions is difficult to achieve as the virus can be delivered as free virions or associated with different lipoproteins and immunoglobulins [545, 546]. Additionally, the presence of quasispecies with variable epitopes in the virus population is likely to contribute to evasion of the humoral response. Nevertheless, neutralisation of a proportion of incoming HCV virions might lead to a reduced viral load allowing the development of CMI in due time, promoting viral clearance. The data in Chapter 6 also highlighted the importance of selecting and/or combining appropriate immunogens in an optimised regimen to induce efficient immunity. The E1/E2-IMX313P DNA prime/ E1E2 recombinant protein boost vaccine strategy is consistent with current vaccination schemes involving priming with DNA and boosting with a recombinant protein or viral vectors that have emerged as the favoured approach to generate protective responses against many diseases, including HCV, HIV, malaria and cancer [547, 548]. More importantly, the homologous DNA prime/DNA boost regimens elicited a NAb response similar to that achieved by boosting with recombinant proteins (Chapter 6) suggesting that the homologous DNA vaccination regimens are equally effective at inducing NAbs as boosting with E1E2 protein.

Although no DNA vaccines have thus far been licensed for human use, many have been licensed for veterinary use [477]. DNA vaccines show no significant side effects in immunised individuals and therefore are regarded as safe [1, 477]. A recent phase 1, open-label first-in-human trial of the candidate tuberculosis vaccine MVA85A-IMX313 in healthy adults demonstrated that the vaccine was well tolerated and immunogenic [426]. Moreover, no auto-immunity was detected towards the oligomerisation domain of mouse [461] or human C4bp [426, 461]. This suggests that it is highly unlikely that auto-immunity towards IMX313/P will be induced by the vaccines used in this thesis. However, if this vaccine is to progress to clinical trials, it will be necessary to perform a preclinical study demonstrating that the immunogenicity of the DNA prime/DNA boost, DNA prime/E1E2 protein boost or DNA prime/HCV VLP boost regimens translate well in larger animal models (such as pigs, see section 7.4.5), and is safe.

It is worth noting that the vaccine immunogenicity increased with the number of doses, suggesting that similar vaccine regimens might be required for p-tE1/tE2-IMX313P to be effective in larger animals or indeed in humans, and this might result in non-compliance. Thus, to prevent this, the delivery of these DNA vaccine should be optimised to increase the vaccine

effectiveness with fewer doses and this could result in further improvements in the immune responses generated by these vaccine constructs. Unfortunately, due to resource and time limitations, examination of the neutralising abilities of serum at various time points throughout the vaccination schedule was not performed. Clearly, further investigation is warranted to comprehensively address this crucial aspect of HCV vaccine development.

Collectively, these results have important implications for the development of anti-HCV preventive vaccines, and they exemplify the flexibility and unique ability of the E1/E2-IMX313/P strategy to generate HCV-specific immune responses after homologous and heterologous prime-boost immunisation.

7.4 Future studies

The results presented in this thesis support further testing of p-tPA-tE1/tE2-IMX313P both in mice and larger animal models (such as pigs or non-human primates). The results from these future studies will be crucial for the future evaluation of these vaccine in human clinical trials.

7.4.1 Assessing the long-term immunity of E1/E2IMX313/P DNA vaccines

This thesis did not investigate certain key aspects related to the immune responses generated following vaccination with p-tPA-tE1/tE2-IMX313P due to unavoidable constraints. For example, long term vaccine experiments were not conducted due to time constraints. These studies could have provided valuable information on the longevity of the E1E2-specific immunity in vaccinated mice. It is thus important to demonstrate that this desirable immunological characteristic is achieved in future studies.

7.4.2 A DNA vaccine strategy to induce HCV-specific NAbs and CMI

No clear correlates of protection against HCV have been defined so far. However, it is generally accepted that an effective HCV-vaccine should elicit both a potent humoral response able to neutralise virus as well as a strong, broad CMI, to limit virus amplification and spread [279, 549] [195, 200, 550]. The Gowans laboratory has developed a novel a DNA vaccine capable of inducing strong CD4+ and CD8+ T cell responses against non-structural proteins 3, 4A, 4B, and 5B [414, 551] similar to those observed during resolution of acute HCV infection. Further experiments should be conducted to assess whether the DNA vaccine constructs reported in this thesis in combination with the DNA vaccine encoding NS proteins can elicit both anti-E1E2 NAb and CMI responses to the NS proteins. This strategy will ensure that the induced antibodies will either prevent HCV infection of host cells or reduce the number of HCV-infected hepatocytes. A T cell response to NS proteins could in turn detect and eliminate virus-infected cells through their effector functions. For example many licensed flavivirus vaccines, such as those against yellow fever virus (YFV), Japanese encephalitis virus (JEV), and tick-

borne encephalitis virus (TBEV), have been shown to elicit strong CMI responses in addition to antibodies [552-555]. The live-attenuated 17D yellow fever vaccine, in particular, can induce strong CD4 and CD8 T cell responses that correlate with antibody responses [556] and are also likely to contribute to the high level of protection afforded by this vaccine.

7.4.3 Generating a multigenotype HCV vaccine

Previous studies have demonstrated that vaccination can induce NAbs to HCV, however, the protection was restricted to homologous virus and was ineffective against heterologous virus challenge [283]. The induction of NAbs against highly variable viruses or different genotypes remains a significant challenge in HCV vaccine research. Nevertheless, recent studies have demonstrated that NAbs can efficiently protect humanised mice and chimpanzees against heterologous virus in passive transfer experiments [142, 152, 192], suggesting that it may be possible to develop a vaccine with broad coverage of HCV genotypes. The induction of a broad CMI may also be useful in mediating protection against homologous HCV infection, but may be seminal to protect against infection with heterologous HCV strains [557, 558]. Therefore, a vaccine covering viral heterogeneity (including different genotypes and subtypes) able to induce a strong NAb response and a vigorous multispecific, broad CMI may be necessary to overcome viral immune escape [559, 560]. Recently, a trivalent vaccine containing soluble E2 from genotype 1a, 1b and 3a elicited pangenotypic NAbs in mice and rhesus monkeys, which neutralised HCVcc of all the seven genotypes more potently than the monovalent counterpart [561]. Furthermore, the NAb generated acted synergistically to inhibit HCV infection. Consequently, the development of a multigenotypic vaccine may be best achieved by exploiting the advantages of DNA vaccination, by encoding multiple antigens from different HCV strains. In such a study, a DNA vaccine encoding consensus HCV antigens or a cocktail vaccine of individual DNA encoding antigens from the main HCV genotypes could be evaluated for their immune potential.

7.4.4 Improving DNA vaccine delivery

Cellular uptake of naked DNA is inefficient *in vivo*, requiring administration of large amounts of DNA. [562]. It is believed that the bulk of DNA injected by the intramuscular route remains extracellular [563]. Several doses of the DNA constructs generated in this thesis were required for effective vaccination. It is likely that similar vaccine regimens will be required for p-tE1/tE2-IMX313P to be effective in larger animal models or humans. DNA vaccines have thus far only been licensed for veterinary use. All licensed DNA vaccines are administered as an intramuscular injection of naked DNA, which is the simplest method of DNA vaccine delivery [477].

Most common alternative strategies to increase the efficiency of DNA delivery *in vivo* include physical delivery methods such as gene gun delivery and electroporation. The Gene gun method uses accelerated particle carrier biocompatible heavy metals to deliver the DNA into the target cell and tissues [341, 342]. However, particle bombardment is restricted to local tissue expression and surgery is often necessary for direct exposure of target tissue. Electroporation uses controlled electrical fields to create pores in the cell membrane that allow uptake of injected DNA into the cell [339, 564]. Despite electroporation presenting an efficient physical DNA delivery system, it results in high cell mortality and the accessibility of electrodes to internal organs is limited. Furthermore, these strategies generally require specialised equipment that might not be universally accessible. Consequently, a convenient simple strategy to deliver DNA effectively *in vivo* is desirable.

Many barriers exist for the efficient transfer of genes to cells, including the extracellular matrix, the endosomal/lysosomal environment, the endosomal membrane, and the nuclear envelope [565]. Over the past decade several strategies have been developed to improve the poor outcome of DNA vaccines by focusing on these obstacles. One such approach designed to overcome the plasma membrane is based on peptide transduction domains (PTDs) (also known as cell penetrating peptides (CPPs)) linked to a dsRNA-binding motif from the dsRNA-binding protein, protein kinase R (PKR) [566]. This study showed that the PTD-dsRNA binding fusion protein (PTD-DRBD) effectively delivered siRNA *in vitro* to a range of primary cells resulting in specific down regulation of different siRNA targets, proving that the siRNA remained functional after delivery by the PTD-DRBD. A siRNA-PTD-DRBD ratio of 1:5 was found to be most effective. Moreover, the siRNA-PTD-DRBD complex was also capable of delivering the siRNA effectively *in vivo* [567]. Oligomers of the HIV-1 Tat protein were shown to have the ability to transfer plasmid DNA into cells [568]. The PTD-DRBD fusion protein contains 3x Tat CPP sequences and a human influenza hemagglutinin (HA)-tag (to follow expression and purification of the protein) fused with a dsRNA-binding protein.

As a potential component of this thesis, I constructed an analogous PTD-dsDBD binding fusion protein that contains 3x Tat CPPs separated by a short linker sequence, the Lambda Repressor helix-turn-helix dsDNA-binding protein which can bind to a specific DNA sequence termed the λ operator or Or2 sequence [569], a V5 tag to permit protein detection, and a 6x His tag for protein purification. Two copies of the OR2 sequence [569] which binds the Lambda Repressor protein are required to be inserted into a DNA vaccine for this strategy to function effectively. Preliminary gel retardation experiments showed encouraging results since the PTD-dsDBD protein was able to bind DNA containing the OR2 sequence and retard the DNA in gel electrophoresis (data not shown). Using a DNA construct encoding LUC, the 2A autoprotease

from FMDV GFP and 2 copies of the OR2 sequence (pCMV-LUC-2A-GFP) would allow the PTD-dsDBD to bind to- and deliver- DNA *in vitro* and *in vivo* as determined by detection of GFP-positive cells using flow cytometry *in vitro* and *in vivo* by direct imaging of LUC in animals [416, 570]. However, preliminary *in vitro* delivery experiments, demonstrated the PTD-dsDBD protein was unsuccessful at facilitating pCMV-LUC-2A-GFP delivery in target cells, evident by the lack on GFP expression (data not shown), even at high DNA: pCMV-LUC-2A-GFP ratios and this work was not pursued.

DNA must enter the nucleus to be transcribed, replicated, and/or integrated, and consequently, the nuclear membrane represents a second major impediment to DNA delivery. Nuclear import of plasmid DNA through the nuclear pore complexes (NPCs) is a sequence-specific process, mediated by specific eukaryotic sequence elements [571]. Plasmids encoding a 72 bp fragment of the SV40 enhancer were shown to target DNA to the nucleus of most cells within several hours, whereas a plasmid lacking this 72 bp sequence either remained in the cytoplasm until cell division, or indefinitely if the cell was nondividing, until it was degraded [571, 572]. This sequence, termed the SV40 DNA nuclear targeting sequence (DTS), has been shown to mediate plasmid nuclear import in all cell lines tested, as well as *in vivo* [573-576]. In addition to encoding 2 copies of the Lambda Repressor binding site, an SV40 DNA nuclear targeting sequence might be incorporated into the DNA vaccine construct to further increase the delivery of DNA in target cells by facilitating their nuclear entry. Furthermore, since many of the DTSs identified to date are derived from cell-specific promoters, this raises the possibility of using these promoters to drive cell-specific nuclear import of the plasmids and transcription of the delivered gene.

7.4.5 Preclinical evaluation of potential HCV vaccine efficacy

Virus challenge represents the only measure of vaccine protective efficacy. Chimpanzees are considered the optimal animal model to test HCV vaccine efficacy as HCV naturally infects only humans and chimpanzees [210]. However, wild chimpanzees are endangered, and the use of captive chimpanzees is expensive and is subjected to stringent ethical consideration, limiting the use of these animals in preclinical trials for candidate HCV vaccines [211, 577]. This necessitates testing candidate HCV vaccines in other alternative small and larger animal models before progression to human clinical trials.

A transgenic mouse model permissive for HCV infection was recently developed [229, 230]. These mice express the HCV entry receptors CD81 and occludin and have been shown to be infected with HCV derived from cell culture and HCV-positive serum, resulting in complete replication of the virus, persistent infection in 80% of the mice and hepatopathological

consequences typical of HCV infection [229, 230]. It would be interesting to examine the viral load in these mice following vaccination with the DNA constructs described in this thesis and examine the ability of the vaccines to protect the mice against challenges with cell culture derived-HCV.

The functions of the major organs, the immune system and body sizes are similar in pigs and humans, making these animals an alternative large animal model to assess the immunogenicity of HCV vaccines [578]. The Gowans laboratory has previously used the pig model to evaluate the immunogenicity of a candidate HCV vaccine [418]. Therefore, it would be useful to examine the immunogenicity of the candidate HCV vaccine described in this thesis in a pig model. Results from these experiments might influence the use of pigs as an alternative cost-effective model to evaluate the immunogenicity of candidate prophylactic HCV vaccines.

7.5 Conclusion

To the best of my knowledge, this thesis reports the first use of the oligomerisation domains IMX313 and IMX313P as a molecular adjuvant to improve the immunogenicity of a E1E2-based HCV DNA vaccine. In summary, with further studies, the DNA-based HCV vaccine regimens described in this thesis have the potential to be considered for future development of a cost-effective, safe and highly effective vaccine against HCV that can be accessed globally and more notably in developing countries where HCV is more prevalent.

Appendices

Appendix I. Primers used for producing the DNA vaccine constructs

Primers used for cloning

Gene of interest primers were used to amplify	**Primer name**	**Primer sequence (5' to 3')** (Lowercase = restriction enzymes, italic = kozak sequence, bold = start or stop codons, underlined = region of homology to gene of interest)	**Restriction Site**
Core	Core fwd	AATAgctagc*GCCACC***ATG**AGCACCAACCCC	NheI
	Core rev	CTTCGgaattc**TCA**GGCGCTGGCAGGAATGGTCAGG	EcoRI
E1	E1 fwd	ACAgctagc*GCCACC***ATG**CACGAAGTGCGGAATGCCAGC	NheI
	E1 rev	TCTGCAgaattc**TCA**TGTCAGTGTGTGGCCGTCCACGCC	EcoRI
E1 (truncated to removed E1 TMD)	E1 rev TC	TCTGCAgaattc**TCA**GTGAGCGCCAGCCACCATATCGATCA	EcoRI
E2	E2 fwd	CTGgctagc*GCCACC***ATG**CACACACTGACAACAGGCGGACATGCCGCCA	NheI
	E2 rev	GTATAgaattc**TTA**GGCCTCGG	EcoRI
E2 (truncated to removed E2 TMD)	E2 rev TC	TCTGCAgaattc**TCA**CCACTTGATGGCGAAGGACACCACG	EcoRI
E2-6×his tag (underlined = 6×his, underlined & bold = E2)	E2-6×His rev	TCTGCAgaattc**TCA**ATGGTGATGGTGATGGTG**CCACTTGATGGCGAAGGACACCACG**	EcoRI
IMX313	IMX313 rev	GGTACCgaattc**TCA**CTCTTTGCTCAGGCCCTGCAGTTCCAC	EcoRI
IMX313P	IMX313P rev	TCTGCAgaattc**TCA**AGACCGCCTCCGCCGTCTGCCTTCCACCTTCAGTTTCTGAATTTCCAGGAA	EcoRI
tPA	tPA fwd	CTGgctagc*GCCACC***ATG**GATGCAATGAAGAGAGGGCTCTGC	NheI

Primers used for fusion/overlapping PCR

Fusion genes primers were used to generate	**Primer name**	**5' to 3' primer sequence**
tPA-E2 (Underlined = tPA, bold = E2)	tPA-E2 ovlp rev	**TGTTGTCAGTGTGTG**GCTAGCCGAAACGAAGAC TGCTCCA
	tPA-E2 ovlp fwd	TTCGTTTCGGCTAGC**CACACACTGACAACAGGCG GACATG**
E2-IMX313 (Underlined = IMX313, bold = E2)	E2-IMX313 ovlp rev	GTCGCCCTGCTTCTT**CCACTTGATGGCGAAGGAC ACCAC**
	E2-IMX313 ovlp fwd	**TTCGCCATCAAGTGG**AAGAAGCAGGGCGACGCC GA
tPA-E1 (Underlined = tPA, bold = E1)	tPA-E1 ovlp rev	**ATTCCGCACTTCGTG**GCTAGCCGAAACGAAGAC TGCTCCA
	tPA-E1 ovlp fwd	TTCGTTTCGGCTAGC**CACGAAGTGCGGAATGCCA GCG**
E1-IMX313 (Underlined = IMX313, bold = E1)	E1-IMX313 ovlp rev	GTCGCCCTGCTTCTT**GTGAGCGCCAGCCACCATA TCGATC**
	E1-IMX313 ovlp fwd	**GTGGCTGGCGCTCAC**AAGAAGCAGGGCGACGCC GA

Primers used for sequencing

Gene of interest	**Primer name**	**Primer sequence (5' to 3')**
BGH poly A	BGH poly A rev	CCATAGAGCCCACCGCATCCCCAGCATGC C
CMV	CMV enhancer fwd	GCGATGTACGGGCCAGATATACGCGTT
	CMV promoter fwd	GTGATGCGGTTTTGGCAGTACATCAATGG G
Kanamycin	KAN resistance rev	TCAGAAGAACTCGTCAAGAAGGCGATAGA AGGCGA

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