Pre-Clinical Evaluation of a Vaccination Strategy to Induce Liver Resident Memory T Cells against Hepatitis C Virus

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Declaration

Acknowledgments

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Abstract
Hepatitis C virus (HCV) is a significant contributor to the global burden of disease with at least 71 million individuals persistently infected with the virus. Although effective anti-viral therapies are available, they are prohibitive in cost and at least 80% of infected individuals are undiagnosed for HCV making the development of an effective vaccine a crucial requirement to eliminate HCV infections. There is much conjecture about how to design an effective HCV vaccine, nonetheless, there is evidence to suggest that a vaccine which can elicit T cell immunity in the liver will be protective. Firstly, tissue-resident memory CD8\(^+\) T (T\(_{RM}\)) cells have been reported to patrol the liver sinusoids for the lifespan of the individual and play vital roles in the elimination of malaria parasites (Plasmodium berghei) following invasion of hepatocytes. Secondly, CD4\(^+\) and CD8\(^+\) T cell-mediated immunity against non-structural (NS) proteins of HCV correlate with recovery from acute infections in patients. DNA and adeno-associated virus (AAV) have an excellent safety profile and have been effective in gene therapy and vaccinations in humans. Our laboratory has also shown that a DNA vaccine encoding HCV NS proteins and a cytolytic protein, perforin (PRF), is more effective than a canonical DNA vaccine in eliciting HCV-specific T cell responses against NS proteins of HCV. Thus, using a DNA vaccine encoding NS5B from HCV genotype 3a and PRF (pVAX-NS5B-PRF) and recombinant AAV (rAAV) encoding the vaccine antigen (rAAV-NS5B-2A-eGFP) vaccination regimens designed to elicit HCV-specific T cell mediated immunity in the liver were developed and evaluated in this thesis. The vaccination strategy involved intradermal (ID) vaccination of mice with pVAX-NS5B-PRF to prime T cells followed by intravenous (IV) vaccination with rAAV-NS5B-2A-eGFP to trap the primed cells.

To achieve the aims of this thesis, an established fluorescent target array (FTA) technology was modified to identify immunodominant CD4\(^+\) T cell and CD8\(^+\) T cell epitopes of NS5B in vivo to thoroughly analyse NS5B-specific T cell responses in vaccinated mice in vivo. Based on the in vivo epitope mapping analysis, a NS5B\(_{451-459}\) tetramer was developed that was crucial for isolating
NS5B-specific CD8+ T cells in vaccinated BALB/c mice and determining whether cells had differentiated into TRM (i.e. CD69+ CD62L-) cells in the liver. rAAV-NS5B-2A-eGFP capable of transducing hepatocytes in vivo was also developed and used in vaccination studies.

The vaccination regimen described in this thesis elicited robust NS5B-specific killing responses by CD8+ T cells and helper CD4+ T cell responses. Importantly, the heightened immunogenicity of the vaccination regimen also correlated with the formation of NS5B-specific CD8+ TRM cells in the liver. Although, the protective efficacy of this regimen still needs to be evaluated following an authentic HCV challenge, this study highlights that the prime/trap regimen is a promising strategy that can be used to develop a protective vaccination regimen against HCV in the future. Moreover, the results bridge an important gap in the HCV vaccine development especially for vaccines that aim to elicit protective T cell responses in the liver to eliminate the virus shortly after infection and before a divergent quasi-species emerges.
**Declaration**

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, 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 a submission in my name, 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.

I give permission for the digital version of my thesis to be made available on the web, via the University’s digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

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Zelalem Addis Mekonnen
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<tbody>
<tr>
<td>AAP</td>
<td>Assembly activating protein</td>
</tr>
<tr>
<td>AAV</td>
<td>Adeno associated virus</td>
</tr>
<tr>
<td>AAVS1</td>
<td>Adeno associated virus integration site-1</td>
</tr>
<tr>
<td>Ad</td>
<td>Adenovirus</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>HLA</td>
<td>Human leucocyte antigen</td>
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<tr>
<td>ART</td>
<td>Antiretroviral therapy</td>
</tr>
<tr>
<td>BGHpolyA</td>
<td>Bovine growth hormone poly adenylation</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase recruitment domain</td>
</tr>
<tr>
<td>CAT</td>
<td>Chloramphenicol acetyl transferase</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxy fluorescein succinimidyl ester</td>
</tr>
<tr>
<td>ChAd</td>
<td>Chimpanzee adenovirus</td>
</tr>
<tr>
<td>CLA</td>
<td>Cutaneous lymphocyte associated antigen</td>
</tr>
<tr>
<td>CLDN1</td>
<td>Claudin 1</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CPD</td>
<td>Cell proliferation dye</td>
</tr>
<tr>
<td>CTV</td>
<td>Cell trace violet</td>
</tr>
<tr>
<td>DAA</td>
<td>Directly acting antivirals</td>
</tr>
<tr>
<td>DAPI</td>
<td>Diamidino phenylindole</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECMV</td>
<td>Encephalomyocarditis virus</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra acetic acid</td>
</tr>
<tr>
<td>eGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>eIF</td>
<td>Eukaryotic initiation factor</td>
</tr>
<tr>
<td>ELISpot</td>
<td>Enzyme-linked immunosorbent spot</td>
</tr>
<tr>
<td>EphA2</td>
<td>Ephrin receptor A2</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FMDV2A</td>
<td>Foot-and-mouth disease virus 2A protease</td>
</tr>
<tr>
<td>FTA</td>
<td>Fluorescent target array</td>
</tr>
<tr>
<td>FV620</td>
<td>Fixable viability dye</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycans</td>
</tr>
<tr>
<td>GMFI</td>
<td>Geometric mean fluorescent intensity</td>
</tr>
<tr>
<td>gt</td>
<td>Genotype</td>
</tr>
<tr>
<td>hAAT</td>
<td>Human (\alpha)-1 anti-trypsin</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks balanced salt solution</td>
</tr>
<tr>
<td>HBV</td>
<td>Hepatitis B virus</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<td>------------------------------------------</td>
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<tr>
<td>HCC</td>
<td>Hepatocellular carcinoma</td>
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<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
</tr>
<tr>
<td>HCVcc</td>
<td>Cell culture derived HCV</td>
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<tr>
<td>HCVpp</td>
<td>HCV pseudo-particle</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethyleneepiperazine-N'-2ethanesulphonic acid</td>
</tr>
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<td>HEK293</td>
<td>Human embryonic kidney cells</td>
</tr>
<tr>
<td>hFIX</td>
<td>Human coagulation factor IX</td>
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<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<td>HPV</td>
<td>human papilloma virus</td>
</tr>
<tr>
<td>HSC</td>
<td>Hepatic stellate cells</td>
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<td>HSPG</td>
<td>Heparan sulfate proteoglycan</td>
</tr>
<tr>
<td>Huh-7</td>
<td>Human hepatoma cells</td>
</tr>
<tr>
<td>ICS</td>
<td>Intracellular cytokine staining</td>
</tr>
<tr>
<td>ID</td>
<td>Intradermal</td>
</tr>
<tr>
<td>IF</td>
<td>immunofluorescence</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal ribosomal entry site</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
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<tr>
<td>ISG</td>
<td>Interferon stimulated gene</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ITR</td>
<td>Inverted terminal repeat</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>JAK/STAT</td>
<td>Janus kinase/signal transducer and activator transcription</td>
</tr>
<tr>
<td>JFH</td>
<td>Japanese fulminant hepatitis</td>
</tr>
<tr>
<td>KC</td>
<td>Kupffer cells</td>
</tr>
<tr>
<td>LamR</td>
<td>Laminin receptor</td>
</tr>
<tr>
<td>LAV</td>
<td>Live attenuated vaccine</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LRR</td>
<td>Leucocyte rich repeat</td>
</tr>
<tr>
<td>LSEC</td>
<td>Liver sinusoid endothelial cells</td>
</tr>
<tr>
<td>MAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAD</td>
<td>Melanoma differentiation antigen</td>
</tr>
<tr>
<td>MAVS</td>
<td>Mitochondrial antiviral signaling</td>
</tr>
<tr>
<td>MCM</td>
<td>Minichromosomal maintenance complex</td>
</tr>
<tr>
<td>MCS</td>
<td>Multiple cloning site</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>miRNA</td>
<td>Micro RNA</td>
</tr>
<tr>
<td>MLTF</td>
<td>Major late transcription factor</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>MVA</td>
<td>Modified vaccinia Ankara</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response gene 88</td>
</tr>
<tr>
<td>NAb</td>
<td>Neutralising antibodies</td>
</tr>
<tr>
<td>NBD</td>
<td>Nucleotide binding domain</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NI</td>
<td>Nucleotide inhibitors</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>NLT</td>
<td>Non-lymphoid tissue</td>
</tr>
<tr>
<td>NNI</td>
<td>Non-nucleotide inhibitor</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide oligomerisation domain</td>
</tr>
<tr>
<td>NPC1L1</td>
<td>Cholesterol transporter Niemann-pick C-1 like 1</td>
</tr>
<tr>
<td>NS</td>
<td>Non-structural proteins</td>
</tr>
<tr>
<td>OCLN</td>
<td>Occludin</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethyleneimine</td>
</tr>
<tr>
<td>PfSPZ</td>
<td>Radiation-attenuated <em>P. falciparum</em> sporozoites</td>
</tr>
</tbody>
</table>
PI  Protease inhibitors
PKR  Protein kinase R
PRF  Perforin
PRR  Pattern recognition receptor
rAAV  Recombinant adeno associated virus
Rag2  Recombination activating gene
RAS  resistance-associated substitution
RBE  Rep binding element
RBS  Rep binding site
RDRP  RNA dependent RNA polymerase
RIG-I  Retinoic acid inducible gene-1
RNA  Ribonucleic acid
ROS  Reactive oxygen species
rpm  Revolution per minute
RSV  Respiratory syncytial virus
RT  Reverse transcriptase
S1PR-1  Sphingosine-1 phosphate receptor-1
scAAV  Self-complementary AAV
SC  Subcutaneous
SCID  Severe combined immunodeficient
SDS  Sodium dodecyl sulfate
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>SEAP</td>
<td>Secreted alkaline phosphatase</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<tr>
<td>siRNA</td>
<td>Short interfering RNA</td>
</tr>
<tr>
<td>SLO</td>
<td>Secondary lymphoid organ</td>
</tr>
<tr>
<td>SR-BI</td>
<td>Scavenger receptor class B type 1</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes simplex virus</td>
</tr>
<tr>
<td>STING</td>
<td>Stimulators of interferon gene</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator transcription</td>
</tr>
<tr>
<td>SURE</td>
<td>Stop unwanted rearrangement event</td>
</tr>
<tr>
<td>SVR</td>
<td>Sustained virologic response</td>
</tr>
<tr>
<td>TBE</td>
<td>Tis borate EDTA</td>
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<tr>
<td>Tbet</td>
<td>Transcription factor T-bet</td>
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<tr>
<td>T&lt;sub&gt;CM&lt;/sub&gt;</td>
<td>Central memory T cells</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>T&lt;sub&gt;EM&lt;/sub&gt;</td>
<td>Effector memory T cell</td>
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<tr>
<td>Tfr1</td>
<td>Transferrin receptor 1</td>
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<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
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<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
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<tr>
<td>TRIF</td>
<td>Toll IL-1 receptor domain containing adaptor inducing-β</td>
</tr>
<tr>
<td>T&lt;sub&gt;RM&lt;/sub&gt;</td>
<td>Tissue resident memory T cell</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
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<td>------------------------------------------------------</td>
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<tr>
<td>trs</td>
<td>Terminal resolution site</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>VARNA</td>
<td>Virus associated RNA</td>
</tr>
<tr>
<td>vgc</td>
<td>Viral genome copies</td>
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<tr>
<td>VLP</td>
<td>Virus like protein</td>
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<tr>
<td>VP</td>
<td>Viral protein</td>
</tr>
<tr>
<td>WPRE</td>
<td>Woodchuck hepatitis virus post transcriptional element</td>
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Chapter 1: Literature Review

1.1 Hepatitis C virus (HCV)

1.1.1 Biology and Epidemiology of HCV

HCV is one of the leading causes of mortality and morbidity in the world. It was initially identified as the causative agent of non-A, non-B hepatitis. HCV is a RNA virus classified in the genus Hepacivirus in the Flaviviridae family [1-3] and is transmitted via different routes including exposure to infected blood, injection drug use, sexual contact and as a result of occupational exposure [4, 5].

An estimated 100 million people are serologically positive for HCV and at least 71 million people are persistently infected with the virus [6, 7]. The most recent estimates suggest that nearly 400,000 HCV-related deaths occur every year, which is an increase by 22% compared to the number of deaths reported annually since the year 2000 [7, 8]. In the next 20 years, though incidence rates are decreasing in developing countries, deaths from HCV-related liver disease will continue to increase [2].

In Australia, 233,490 people were estimated to be persistently infected with HCV in 2013 and 530 people died from HCV-related liver disease. In Australia, costs related to HCV are estimated to increase from $224 million/year in 2013 to $305 million/year in 2030 [9]. Approximately 10,000 new HCV infections occur in Australia per year [10]. However, as a result of AUD$ 1 billion commitment from the Australian government all HCV infected individuals have access to treatment [11].

HCV is classified into different genotypes based on phylogenetic and sequence analysis of the viral genome. Until the recent discovery of a novel HCV genotype, genotype 8 [12], HCV strains were classified into seven genotypes (1-7) and 67 confirmed and 20 provisional subtypes. The different genotypes differ by 30-35% in the nucleotide sequence while the subtypes differ in less
than 15% in the nucleotide sequence [2, 13]. Globally, genotype 1 is the most prevalent, accounting for 46.2% of infections followed by genotype 3 which accounts for 30.1%. Genotypes 2, 4 and 6 account for the remaining cases [2]. Genotype 7 was identified in Canada in four individuals originating from Democratic Republic of Congo [14]. Genotype 8 was also identified in Canada from four patients who were originally from Punjab, India [12].

Acute HCV infection is clinically mild and typically unrecognized as it results in non-specific flu-like symptoms that are commonly observed in most viral infections. Patients who are HCV RNA positive for six months or longer after the acute infection are defined as persistently infected. The transition from acute to chronic hepatitis C is usually sub-clinical and is missed as only 16% of patients are symptomatic [15].

Twenty-five percent of patients will clear the infection spontaneously within a few months of infection, but the virus remains persistent in 75% of the infected individuals [16]. Persistent HCV infection is associated with the development of liver cirrhosis, hepatocellular cancer and liver failure [2].

Several viral and host factors determine the outcome of the infection [15, 17]. A prospective study that assessed clinical, virological, genetic and immunological parameters of individuals with acute HCV infection indicated that multi-specific T-cell responses, raised levels of alanine aminotransferase (ALT) that decline within 4 weeks, jaundice, female gender and > 2.5 log10 HCV-RNA that drops within 8 weeks were associated with spontaneous virus clearance [18]. Genome wide association studies (GWAS) showed a correlation between a single nucleotide polymorphism (SNP) in different alleles and spontaneous- or treatment induced- virus clearance [19, 20]. A SNP (rs2596542) in MHC class I poly-peptide related sequence A (MICA) was significantly associated with HCC in 721 HCV-positive cases and 2890 HCV-negative controls from a Japanese population [19]. Kamal, et al also showed that polymorphism in IL-28B, a
member of the IFN-λ family, was associated with strong anti-HCV T cells responses. [18]. Similarly, a meta-analysis of host polymorphisms reported that $DQBI^*02$, $DQBI^*0301$, $DRBI^*04$ and $DRBI^*11$ were HLA associated polymorphisms correlated with spontaneous- or treatment induced- HCV clearance [21, 22].

1.1.2 HCV genome organisation and replication

1.1.2.1 Genome organisation
The HCV genome (Figure 1.1) contains a single open reading frame (ORF) between untranslated regions (UTR) that are found at the 5’ and 3’ ends. The internal ribosome entry sites (IRES), that is contained in the 5’ end of UTR is responsible for the cap-independent initiation of translation [23].

![Image of HCV genome and polyprotein processing](https://via.placeholder.com/150)

**Figure 1.1: The HCV genome and polyprotein processing.** The HCV RNA genome (top) contains one long ORF flanked by 5’ and 3’ UTRs. IRES-mediated translation of the ORF leads to a polyprotein (bottom) that is co- and post-translationally processed into ten viral proteins [24].
In cap-independent IRES-mediated translation, viral protein expression is regulated by direct recruitment of ribosomes to the start site of translation. Translation is initiated when a vacant 40S ribosomal subunit binds to the IRES. This binary complex then binds to eukaryotic initiation factor (eIF) 3, as well as the ternary complex eIF-2: Met–tRNAi: GTP to form a 48S-like complex which subsequently leads to the formation of the 80S complex and hence the formation of the first peptide [3]. The HCV RNA 5’UTR contains two conserved microRNA (miR)-122 sites; miR-122 is abundant in human hepatocytes [24]. miR-122 binding has a stimulatory effect on translation, protects degradation of uncapped HCV RNA and inhibits innate immune recognition of the HCV genome [25-27].

1.1.2.2 HCV attachment and entry to the cell
The process that leads to HCV infection of a cell is a complex process that involves attachment then entry and fusion of HCV with its target cells. Glycoproteins on the virus surface and specific receptors on the target cell determine the interaction between HCV and target cells [28].

The binding of HCV E2 to glycosaminoglycans (GAG) on hepatocytes may be the first step in the interaction. This interaction is facilitated by heparan sulfate proteoglycans (HSPG) expressed on hepatocytes. Barth and colleagues showed that degradation of cell surface HSPG with heparinase resulted in a marked reduction in viral envelope protein binding to hepatocytes [1, 29]. In addition to the GAG, lipoprotein receptors and viral lipoprotein structures were shown to play a role in the process of HCV attachment to hepatocytes. ApoE associated with HCV particles is involved in the attachment of HCV to hepatocytes and the addition of HCV to Huh-7 cells in the presence of ApoE-specific monoclonal antibodies (MAb) prevented virus attachment [30]. However, when the anti-ApoE antibodies were added after virus attachment viral protein expression was not inhibited suggesting that ApoE contributes to virus attachment rather than virus entry [30-32]. Low density lipoprotein receptors (LDL) were also shown to act as a
cooperative HCV co-receptor that supports viral entry and infectivity through an interaction with ApoE [33, 34].

Once attached to hepatocytes, HCV is internalized via pH dependent clathrin-mediated endocytosis [1]. The entry process requires different molecules including the tetraspanin, CD81, scavenger receptor class B type I (SR-BI), claudin-1 (CLDN1), occludin (OCLN) and the cholesterol receptor Niemann-Pick C1-like 1 (NPC1L1) [35]. Other factors like epidermal growth factor receptor (EGFR), ephrin receptor A2 (EphA2) and transferrin receptor 1 (TfR1) are also involved in the entry process [36].

CD81 is a member of the tetraspanin membrane protein superfamily, whose members function to organize signaling complexes at the cell surface by association with other tetraspanins, integrins, and signaling proteins in a cell-type-dependent manner [37]. CD81 is a HCV co-receptor that directly binds to HCV E2 [35] and plays a key role in HCV entry process [38]. Zhang et al. showed the role of CD81 i) by blocking infection using anti-CD81 MAb and ii) by using short interfering RNA (siRNA) that suppressed expression of CD81 in Huh-7.5 cells [39]. Incubation of target cells in the presence of MAb against CD81 followed by infection with HCV pseudoparticles (HCVpp) inhibited infection of Huh-7.5, Hep3B and PLC/PR5 cells [39]. A greatly reduced level of infection was also observed when siRNA that specifically target CD81 (siCD81) was introduced to Huh-7.5 cells. From both observations, it was concluded that CD81 is important for HCV entry to target cells [39].

SR-BI is a lipoprotein receptor of 509 amino acids (aa) with cytoplasmic N- and C-terminal domains separated by a large extracellular domain. It is expressed primarily in liver and steroid hormone producing (steroidogenic) tissues, where it mediates selective cholesteryl ester uptake from high-density lipoprotein (HDL) and may act as an endocytic receptor [40]. SR-BI was identified as a co-receptor by its ability to bind recombinant HCV E2 on HepG2 cells which lack CD81 expression [41]. Zeisel et al. reported that antibody-or siRNA-mediated inhibition of SR-
B1 resulted in reduced susceptibility of human hepatoma cells to cell culture grown HCV (HCVcc) [42]. In another study, it was shown that mutant SR-BI with reduced binding capacity for soluble E2 (sE2) was unable to restore susceptibility of SR-BI knocked down Huh-7.5 cell lines to HCVcc infection [40].

OCLN, an endogenous protein expressed in both HCV-permissive and -non-permissive cells, is another important protein required for HCV entry. Although its over expression failed to change the susceptibility of cells to HCVpp, silencing of OCLN inhibited HCVpp and HCVcc infection of Huh-7 cells [43].

A study that demonstrated the role of CLDN-1 as a viral entry factor used non-permissive cells to confirm its role [44]. In this study, expression of CLDN-1 in HEK-293 cells increased their susceptibility to lentiviral particles expressing HCV glycoproteins, HCVpp and HCVcc. Antibody-mediated blockade of CLDN-1 in these cells prevented viral entry suggesting a role for CLDN-1 in viral entry [44].

The NPC1L1 cholesterol uptake receptor which is expressed on hepatocytes was also recognized as an HCV entry factor. Down regulation or antibody-mediated blocking of NPC1L1 resulted in a reduced susceptibility of Huh-7 cells to HCVcc. In addition the inhibition was HCV-specific as a similar effect was not observed after vesicular stomatitis virus G-protein pseudotyped particle (VSVGpp) infection [45].

The entry processes which involves the above factors eventually lead to release of the HCV genome into the cytoplasm, where primary translation can occur [24, 46].

1.1.2.3 RNA replication and protein translation

Once in the cytoplasm, the HCV RNA functions directly as mRNA, because it is a positive sense RNA, and protein translation is initiated through the IRES in the 5’UTR [23, 47]. Following polyprotein cleavage, the viral RNA and non-structural (NS) proteins form a membrane-associated replication complex [47, 48]. Viral RNA replication occurs in this replication complex
using the positive strand RNA genome as a template for the NS5B polymerase to generate the negative strand replicative intermediate [1]. Nascent positive strand RNA genomes can be further translated to produce new viral proteins, serve as templates for further RNA replication or be assembled into infectious virions [46, 49].

Following RNA replication nascent HCV particles form by budding into the endoplasmic reticulum (ER) where the viral core protein, E1, E2 and the viral genome assemble [35, 49]. Nascent virus particles leave the cells through the secretory pathway [50, 51].

Protein translation results in a poly-protein that is cleaved into mature proteins, structural (Core, E1 and E2) and NS proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B), by viral and host proteases [1, 3].

HCV core is the first structural protein encoded by the HCV ORF and forms the viral capsid [52]. Host signal peptidase cleaves core from the polyprotein and leaves it embedded in the ER while the host peptide peptidase cleaves it into p21 and p19 releasing p21 from the ER [52, 53]. HCV core has numerous functions involving RNA binding, immune modulation, cell signaling, oncogenic potential and autophagy [1]. Core plays an important role in the pathogenesis of HCV because of its ability to 1) circumvent innate immune response by down regulating signal transducer and activator transcription 1 (STAT1) which is required for the formation of IFN-stimulated gene (ISG) factor 3 (ISGF3) and 2) its ability to inhibit priming of CD4+ and CD8+ T cells [54, 55].

The envelope proteins, E1/E2, are glycosylated envelope glycoproteins that surround the viral particles [1]. These proteins are required for viral entry and hence are the targets for neutralising antibodies (NAb). However, the variability of these proteins means that HCV can escape from NAb [56, 57].

The NS proteins are processed by two viral proteases; the NS3 serine protease and the NS2 cysteine protease [52]. NS3 serine protease mediates autocleavage of NS4A and NS4B, after
which NS4A associates with the N-terminus of NS3. The resulting NS3/4A protease complex can then cleave at the NS4B/5A and NS5A/5B junctions, whereas the cleavage between NS2 and NS3 is mediated by the NS2 cysteine protease [3, 46].

Together, the NS proteins constitute the viral proteins of the replication machinery, which replicates the positive sense RNA genome through a dsRNA replicative form and negative strand intermediate. The roles of each of these NS proteins is summarised in Table 1.1.

1.1.3 Pathogenesis of HCV

HCV primarily infects liver cells and demonstrates high levels of virus production, with a production rate of approximately $10^{12}$ particles per day in a single infected individual [58], exceeding that of human immunodeficiency virus (HIV) and hepatitis B virus (HBV) [59]. Assuming 10% of hepatocytes from $2 \times 10^{11}$ hepatocytes per liver tissue are actively producing HCV virions, each hepatocyte will produce approximately 50 viral particles per day [60, 61].

Initial infection with HCV is characterized by the detection of virus in the blood within 2–14 days of exposure, increases in the levels of liver-associated serum enzymes (i.e., ALT and aspartate aminotransferase (AST)) and the gradual appearance of HCV-specific antibodies within 20–150 days of exposure [62]. Patients who cleared the virus during the acute phase showed higher levels of viral RNA and ALT during the first two months after infection with subsequent decline in the third and fourth months [63, 64]. Patients who present with jaundice (thus indicating more severe liver injury) during primary infection are more likely to clear HCV than the majority who acquire HCV without apparent symptoms [65, 66]. More specific symptoms of viral hepatitis can be encountered in a minority of individuals: jaundice, dark urine, anorexia, aversion to smoking among smokers and abdominal discomfort may occur. Physical findings are usually minimal, apart from jaundice in a third of patients. Chronic hepatitis is the most common outcome, usually characterized by raised serum aminotransferases and may lead to fibrosis and cirrhosis in the liver. Thus chronicity is the major complication of acute hepatitis C [15].
<table>
<thead>
<tr>
<th>NS protein</th>
<th>Function</th>
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<tr>
<td>p7 viroporin</td>
<td>Essential for the production of infectious virus [67]; increases cell permeability, reduces acidification of intracellular vesicle and impairs glycoprotein trafficking [68, 69]</td>
</tr>
<tr>
<td>NS2</td>
<td>NS2 is essential for productive viral infection [70]; immunosuppressive; down regulate IFN [71], cysteine protease that cleaves at NS2/NS3 junction [72]</td>
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<tr>
<td>NS3</td>
<td>Serine protease activity in the N-terminal region and NTPase/RNA helicase activity in the C-terminal region [73]; requires C-terminal portion of NS2 (NS2/3 protease) to cleave at NS2-NS3 junction [74]; requires NS4A (NS3/4A protease) for its protease activity to cleave NS3-4A junction [73]; has an important role in supressing host anti-viral response by acting on Toll-IL-1 receptor domain containing adaptor inducing INF-(\beta) (TRIF) and mitochondrial anti-viral signaling protein (MAVS) [75, 76]</td>
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<tr>
<td>NS4B</td>
<td>Alters host cell membrane during HCV replication [77]; inhibits retinoic acid inducible gene-I (RIG-I) mediated IFN-(\beta) production [78]</td>
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<tr>
<td>NS5A</td>
<td>Binds to RNA when phosphorylated to stabilise RNA during replication [79]; reduced NS5A phosphorylation impaired viral production in JFH-1 culture system while it enhanced replication in the gt1b replicon system [80]; Impairs cytokine production via its interaction with Myeloid differentiation primary response gene 88 (MyD88) [81]</td>
</tr>
<tr>
<td>NS5B</td>
<td>RNA dependent RNA polymerase (RDRP) [82]; mediates replication of HCV genome [83]</td>
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Persistent infection with HCV generally results in a slowly progressive disease characterized by chronic hepatic inflammation leading to the development of cirrhosis in approximately 10–20%
of patients over 20–30 years of HCV infection. It is the leading cause of end-stage liver disease, hepatocellular carcinoma (HCC) and liver-related death in the Western world. After the establishment of cirrhosis, the outcome is not predictable; cirrhosis may remain indolent or progress to HCC, hepatic decomposition and death [15]. In the absence of antiviral therapy, 67%-91% of patients with HCV-related liver cirrhosis die due to liver-related causes, including HCC or hepatic failure [84]. The transition from chronic liver disease to cirrhosis involves inflammation, activation of hepatic stellate cells (HSC) with ensuing fibrogenesis, angiogenesis, and parenchymal necrotic lesions caused by vascular occlusion [85]. Cirrhosis can result in major complications of liver failure in patients with chronic HCV infection. However, not all patients with cirrhosis will develop complications [86]. Hepatic decompensation, generally defined as the development of ascites, jaundice, variceal bleeding, and/or hepatic encephalopathy, is another outcome of cirrhosis [84].

HCC is one of the most common malignant tumours, representing more than 5% of all cancers [87]. HCV-associated HCCs develop almost exclusively in patients with liver cirrhosis (> 90%) [88]. The mechanism by which HCV infection causes HCC is not clearly understood. However, HCC may result from chronic necro-inflammatory liver disease or as a consequence of direct oncogenic effect of HCV [87]. Cell damage and chronic inflammation may contribute to the malignant transformation of hepatocytes directly through telomere shortening and inactivation of cell cycle checkpoints, release of reactive oxygen species (ROS), or altering paracrine signaling in the cellular environment [88].

1.1.4 Immune response to HCV

1.1.4.1 Innate immune response to HCV

Innate immune responses are the first line of defence against viral infections. In HCV infection innate immunity is important to control viral dissemination and replication [89, 90]. Pattern recognition receptors (PRRs) within infected cells sense the virus as non-self and induce anti-
viral defences (Figure 1.2) [91]. PRRs include RIG-I, toll-like receptors (TLR), nucleotide oligomerization domain (NOD) like receptors (NLR) and protein kinase R (PKR) [91]. The viral components recognized by these PRRs are specific motifs within the viral products termed pathogen-associated molecular patterns (PAMP) [92]. The interaction between PRRs and PAMPs induces activation of transcriptional pathways that allows rapid activation of the innate immune system [93].

**Figure 1.2: Immune sensing of HCV by PRRs** [91]. During HCV infection, PKR, RIG-I and TLR-3 recognize dsRNA and become activated. Activation leads to the transduction of signaling through MAVS or TRIF, and subsequent phosphorylation and dimerization of IFN regulatory factor-3 (IRF3) to induce the production of IFN-β, IFN-λ, ISGs and proinflammatory cytokines.

MT: Mitochondria; ER: Endoplasmic reticulum; ES: Endosome; PX: Peroxisome
1.1.4.1.1 Innate immune recognition of PAMPs by PRRs

RIG-I belongs to a family of RNA helicases and contains a caspase recruitment domain (CARD) in its N-terminus that allows the recruitment of proteins [94, 95]. RIG-I recognizes blunt or 5′-triphosphorylated (5′ppp) ends of viral genomic RNA segments [94, 96]. Binding of RIG-I to dsRNA leads to signal transmission through the RNA helicase/ATPase domain of the RIG-I. This signaling in turn initiates downstream signaling events that lead to the activation of IRF-3 and nuclear factor kappa B (NF-κB). These signaling cascades induce expression of ISG that control infection [94, 97]. Another RNA helicase that recognises viral RNA is melanoma differentiation antigen 5 (MDA5) [98]. MDA5 mainly recognises long cytoplasmic double stranded (ds) RNAs [99].

TLRs are a family of transmembrane PRR. There are 10 TLRs among which TLR 3, TLR 7 and TLR 9 are associated with virus recognition. TLRs sense virus from within endosomal compartments to signal innate defences. TLR3 recognizes dsRNA, TLR7 detects single stranded (ss) RNA and TLR9 interacts with unmethylated DNA with CpG motifs [98, 100]. TLR3 contains a cytosolic carboxyterminal Toll-IL1 receptor homology (TIR) domain that activates downstream signaling once it recognises dsRNA via its amino terminal ectodomain [91]. Signals from TLR 3 are transduced through the adaptor protein Toll-IL 1 receptor homology (TIR) domain containing TRIF while TLR7 and TLR9 signal through MyD88 and the IRAK4-IRAK1-IKKa kinase cascade. Finally, both pathways lead to the activation of NF-κB and IRF3 [91, 98].

NLRs are cytoplasmic receptors that play a crucial role in the innate immune response by recognizing PAMPs [101]. There are five classes of NLR; NLRA (which contain an acidic transactivation domain), NLRB (contains a baculovirus inhibitor of apoptosis protein repeat (BIR)), NLRC (contains a CARD), NLRP (contains a Pyrin domain) and NLRX (contains an unknown domain) [102]. NLRC has been shown to recognize ssRNA derived from respiratory syncytial virus (RSV), influenza A virus, and parainfluenza virus. Upon recognition, NLRC2
associates with IFN promoter stimulator-1 (IPS-1) through an interaction dependent upon the leucine rich repeats (LRR) and nucleotide-binding domains (NBDs) of NLRC. This leads to the production of type I IFN and proinflammatory cytokine release [103].

1.1.4.1.2 Interaction of PRRs with HCV PAMPs

i. RIG-I: RIG-I recognises HCV PAMPs containing a 5’ppp motif in the genomic RNA [91]. Wang et al. showed the role of RIG-I signaling in the control of HCV infection. They co-cultured HCV-infected Huh-7 cells with HSC; LX-2 stimulated with or without 5’ppp dsRNA to stimulate the RIG-I ligand and observed significant inhibition of HCV replication. They also demonstrated that IFN-β expression was higher in stimulated cells both at RNA level and protein level [104].

ii. MDA5: siRNA mediated knock down of MDA5 in Huh-7 and HepG2 cells resulted in severely diminished level of IFN production when these cells were transfected with IFN-inducing HCV RNA [105, 106]. Recognition of viral RNA by RIG-I or MDA5 induces a conformational change that exposes the N-terminal CARD domains, which interact with the CARD domain of MAVS that leads to downstream signaling pathways of IFN production [94, 107, 108]. MAVS involvement in the IFN signaling pathway was confirmed in a study that used the NS3/4A protease to deplete endogenous MAVS [107]. According to this study Huh-7 cells over expressing NS3/4A showed increased levels of IFN-λ and activation of IRF 3 when transduced with lentiviral vectors encoding protease resistant MAVS [107].

iii. TLR: HCV structural and non-structural proteins are important PAMPs recognised by TLR2, TLR4, TLR7 and TLR9 [109]. It has been shown that HCV derived immunostimulatory RNA oligonucleotides were recognised by TLR7 leading to the activation of IRF7 and NF-κB in Huh-7 cells [110]. Chloroquine-mediated inhibition of TLR7 signaling in dendritic cells (DC) and peripheral blood mononuclear cells (PBMC) as well as siRNA-mediated knock down of TLR7 expression in Huh-7 cells resulted in reduced production of IFN-α and activation of NF-κB respectively [110]. Another study reported that recognition of HCV NS5A by TLR4 resulted in
secretion of IFN-β and IL-6 from B cells and hepatocytes as noted by the inhibition of HCV-induced cytokine secretion when siRNA against TLR4 was introduced [111]. The role of TLR3 has also been described in suppressing HCV replication through IFN-β and ISGs, in non-parenchymal liver cells, mainly liver sinusoid endothelial cells (LSEC) and Kupffer cells (KC) [112].

**iv. NLR:** An interaction between NLR and HCV is not well established and to assess the involvement of NLR in innate immune response against HCV, Vegna *et al.* transduced HepaRG cells with a retrovirus vector encoding HCV NS5B and showed high levels of NOD1 expression from these cells. Expression was specific to NS5B as HCV core, NS3, NS4A and NS5A proteins did not alter NOD1 expression [113]. This study also showed that the catalytic activity of NS5B is important for NOD1 mediated downstream signaling of IL-8 and tumour necrosis factor (TNF)-α production. This was evidenced by the use of mutation induced catalytically inactive NS5B which failed to induce with induce NOD1 expression in HepaRG cells [113]. Amina *et al.* showed that exposure of macrophages to HCV induces IL-1β expression through phagocytic virus uptake that activates MyD88, TLR7 and NLRP3 pathways [114].

### 1.1.4.1.3 HCV and Natural killer (NK) cells

NK cells are important innate immune cells in many viral infections. They recognize and kill virus infected cells and produce antiviral cytokines such as IFN-γ and TNF [115]. When NK cells were cocultured with HCV replicon containing cell lines they were shown to secrete soluble factors which suppress replicon expression and these soluble factors are important in the control of HCV infection [116]. According to this study NK cells did not show a cytolytic effect on 51Cr labelled replicon harbouring cells as no significant release of 51Cr to the medium was observed after 4 hours of incubation [116]. However, this direct lysis inability of NK cell was reversed when NK cells were activated by IL-2 [117]. When constitutively activated IL-2 cDNA transfected NK cells were cocultured with replicon containing hepatoma cell line targets, they
were able to cause lysis of the target cells suggesting that NK cells require IL-2 to be activated [117]. NK cells also showed a positive impact on the clearance of HCV among HIV/HCV co-infected patients [118]. NK cell degranulation was also shown to play role in the cross talk between the innate and adaptive immune response as expression of CD107a, the degranulation marker, correlated with HCV specific T-cell response. However, the correlation was not related to the outcome of HCV infection [119].

1.1.4.1.4 Innate immune evasion by HCV

Although the recognition of HCV PAMPs by PRRs results in immune activation and IFN expression, HCV has the ability to disrupt the PAMP responsive signaling pathways and evade antiviral defences leading to viral persistence [120]. Components of the type I IFN activation pathways including TRIF and MAVS are important host substrates that are targets of HCV NS3/4A protease cleavage [121, 122]. This ultimately blocked the activation of ISG and prevented TLR 3 and RIG-1 signaling [123]. NS4B has also been identified as a strong inhibitor of RIG-I mediated INF-β production as the protein N-terminus was shown to directly bind and inhibit stimulators of IFN genes (STING) activity [124, 125]. NS5A is also among the components of HCV that plays a role in attenuating the innate immune response via its ability to inhibit ISGs 2’-5’ oligonucleotide synthase and induce IL-8 which antagonizes overall ISG expression [126]. Formation of a heterodimer between NS5A and PKR inhibits cyclophilin A activity which in turn blocks IFN effector function [127]. In addition, HCV core protein has been indicated to interfere with Janus kinase/signal transducer and activator transcription (JAK/STAT) signaling to down-regulate ISG expression [128].

HCV has also been shown to inhibit IFN-γ production by NK cells, following cross-linking of CD81 with HCV E2 after exposure to co-stimulatory cytokines [129]. Engagement of CD81 inhibited CD16-mediated tyrosine phosphorylation which is an important signaling pathway to
NK cell activation [130], although this inhibitory activity was not observed when HCV envelope proteins were presented as a component of an infectious HCV particle [131].

1.1.4.2 The adaptive immune response
Unlike the innate immune response that responds within hours or days of infection the adaptive immune response is usually detected 6-8 weeks after infection [89]. The adaptive immune response is associated with recovery from HCV [132].

1.1.4.2.1 Humoral immune response
NAb may directly block attachment of the virus to the host cell and thus inhibit dissemination of infection and may interfere with post-binding steps such as entry of the virus into the host cell. Moreover, NAbs may act as an opsonin in enhancing phagocytosis of virus particles, thereby decreasing viral load [133, 134].

NAb are induced during HCV infection to target epitopes within the HCV envelope glycoproteins E1 and E2, or the E1E2 heterodimer [135]. Pestka et al. reported that rapid induction of NAb was associated with clearance of HCV in a single source out-break of HCV [136]. According to the report, among 49 women who were accidentally exposed to HCV contaminated anti-D immunoglobulin, 23 developed persistent infection and they were characterized by the absence or low titre of NAbs during early phase of infection [137]. A study conducted to assess the role of NAb on the clearance of HCV indicated that both the titre and breadth of NAb was higher among subjects who cleared the virus than those among whom the virus persists [136]. This study also showed that the breadth of NAb response decreases after control of viremia and increased during persistent infection [136], as viral persistence in the presence of NAb was still observed.

A study by Zhang and colleagues showed why such viral persistence is common [138]. According to this study the mechanism of viral persistence is due to interfering antibodies that bind to different epitope sites on the E2 glycoprotein which was evidenced by an increased efficacy and
breadth of NAb when the interfering antibodies were removed [138]. However, the concept of interfering antibodies was challenged by other reports that showed the effectiveness of polyclonal antibodies to protect against HCV infection [139] and the additive effect of murine and human immunoglobulins that target different regions of E2 [140, 141].

There are also other mechanisms by which HCV escapes from NAb, among which the most commonly reported mechanism is mutation [142]. Lack of proof reading by the viral polymerase coupled with high replication capacity results in the generation of many variants that have a potential to escape neutralisation [142, 143]. As a result, antibodies generated at certain stage of HCV infection were unable to neutralise HCVpp cloned from the same patient at different time point [56, 144]. Dowd et al. tested the neutralisation ability of acute phase sera from HCV patients against HCVpp cloned from the same patient at different time point and showed neutralisation against earlier HCVpp was greater than late stage HCVpp [144]. In another similar report, NAb failed to neutralise HCVpp bearing autologous glycoprotein sequences that were present at a given time [56]. Both studies suggest that HCV is exposed to selection pressure from the humoral immune response which ultimately leads to the emergence of antibody-mediated neutralisation-resistant variants.

Another mechanism of HCV to escape antibody mediated clearance is cell-to-cell transmission [142, 145]. When CD81 negative HepaG2 cells were cocultured with Japanese fulminant hepatitis (JFH)-infected cells in the presence of antibodies that can neutralise cell free virus, HepaG2 cells were infected [145]. This showed that HCV can transfer between cells without the involvement of CD81 via a cell-to-cell contact thus preventing virus neutralisation.

1.1.4.2.2 Cell mediated immune response (CMI)
Studies conducted in patients as well as animal models indicated that strong, multi-specific and long-lasting T-cell immune responses play a vital role in the control of HCV infection [132, 146].
Spontaneous eradication of acute HCV infection is associated with the early induction of broad CD4+ and CD8+ T-cell responses to HCV NS proteins 3, 4, and 5, and to a lesser extent, to the HCV core protein [147]. Antibody-mediated depletion of CD8+ memory T cells resulted in prolonged HCV replication in chimpanzees that were otherwise able to rapidly clear the virus [148] while another study reported that antibody-mediated depletion of CD4+ T cells was also associated with viral persistence [149]. In fact the intrahepatic antiviral CD4+ and CD8+ T cell response is associated with acute control of HCV infection [146] and the decrease of the viral titre coincides precisely with the appearance of HCV-specific T cells and IFN-γ expression in the liver, suggesting that viral clearance is T cell mediated [150]. At the time of clinical presentation and ALT elevation, vigorous proliferation of HCV-specific CD4+ T cells with concomitant IL-2 and IFN-γ production is readily detectable in the blood of patients who recover and clear the infection [151-153]. In contrast, HCV-specific CD4+ T cell responses are absent or weak in those who subsequently develop chronic infection [154]. On the other hand, CD8+ T cells are detectable in the blood of acutely infected patients regardless of virological outcome [154]. In a case-control study that assessed the outcome of acute HCV infection, it was shown that spontaneous clearance of HCV was strongly correlated with high frequency HCV specific CD8+ T cells that are transcription factor T-bet positive (T-bet+), an important regulator of the production of IFN-γ and cytotoxic molecules by CD8+ T cells [155-157]. Lechner et al. also studied immune response in acute HCV infection and showed a higher CD8+ T cell response among individuals who cleared the virus [158]. However, failure to sustain a CD8+ T cell response was shown to be associated with progression to virus persistence [159].

For long term protection from chronic HCV infection memory CD8+ T cells play a vital role in chimpanzees which were serially infected with HCV [148]. Following a second infection these animals were able to clear the infection within 14 days and this was associated with increased cytotoxic activity of liver resident CD8+ T cells and expansion of CD8+ and CD4+ cells in the
blood. Antibody-mediated depletion of CD8\(^+\) memory T cells resulted in a failure to resolve a third infection indicating that CD8\(^+\) memory T cells were protective against repeated HCV infection [148]. Another study that compared 17 individuals who developed persistent infection and 14 individuals who cleared infection reported that the breadth of CD8\(^+\) T cells predicted the outcome of infection as patients who demonstrated CTL responses to at least two HCV peptide pools were statistically more likely to contain HCV infection than patients with responses to \(\leq1\) HCV peptide pool [160]. Others also observed that individuals who clear HCV showed significantly broader CTL responses of higher functional avidity and wider variant cross recognition capacity than non-clearers [158, 159, 161].

### 1.1.5 Model systems for HCV study

#### 1.1.5.1 In vitro systems

Several in vitro systems for HCV are important in the study of the viral life cycle, pathophysiology and to test efficacy of antivirals [162]. Some of these models are described in the following paragraphs

#### 1.1.5.1.1 Primary hepatocytes

Primary hepatocytes from humans and chimpanzees were the targets of initial attempts to establish HCV infection. It was possible to detect HCV RNA from these cells when they were infected with HCV-containing serum [163]. Human fetal hepatocytes exposed to serum from HCV patients supported replication of HCV gt 1a, 1b, 2a, 2b, and 3. These cells released HCV to the medium for at least 2 months and expressed HCV core protein and negative strand RNA [164]. In another study it was described that normally differentiated human primary hepatocytes supported productive replication of infectious clones of HCV gt 1a, 1b, and 2a, that, when released to the medium, was able to infect naïve hepatocytes [165]. However, the cells had a short passage life and were prone to contamination which pushed scientists to examine better strategies [163].
1.1.5.1.2 HCV Replicon

The HCV replicon was developed in 1999 and allowed the study of the viral RNA replication in vitro [166]. This HCV replicon was derived from the consensus Con1 cDNA that was isolated from the liver of a patient chronically infected with a genotype 1b strain and is composed of the HCV 5’-UTR fused to the region encoding the first 12 amino acids of the core, the neomycin phosphotransferase (NeoR) gene as a selectable marker, and the HCV NS3-NS5B regions under the control of the encephalomyocarditis virus (EMCV) IRES, followed by the HCV 3’-UTR. This was constructed after removing coding sequences for C, E1, E2 and p7 of the consensus genome Con-1 (Figure 1.3) [166, 167]. Huh-7 cell were transfected with RNA that was transcribed in vitro by T-7 RNA polymerase resulting in the production of cells stably replicating HCV RNA at low levels [5, 163]. HCV replicons have proven to be extremely valuable for studies on the process of HCV replication, as well as for testing of novel antiviral compounds that specifically target the protease activity of NS3 or the polymerase activity of NS5 [168]. The drawback of subgenomic replicons was that even though they express all the NS viral proteins required for viral RNA replication they were unable to produce infectious HCV particles as they are devoid of the coding regions for structural proteins [162].

**Figure 1.3: HCV sub-genomic replicon:** A sub-genomic replicon is derived from the cloned HCV genome by replacing the region that encodes the core protein to the NS2-encoding region of the consensus genome Con-1 by the neomycin phosphotransferase gene (neo) and the IRES of EMCV [169].
1.1.5.1.3 Cell culture grown HCV (HCVcc)
The shortcomings of subgenomic replicons were overcome when JFH-1 clone was isolated which replicated 20-fold higher than the Con-1 replicons as determined by colony formation efficiency in Huh7 cells [162, 170]. JFH-1 is a strain of HCV recovered, with its entire genome, from the serum of a Japanese patient with fulminant hepatitis [171]. Based on this isolate a subgenomic replicon was constructed and tested for its replication ability [170]. Unlike its predecessors, JFH-1 replicon did not require adaptive mutation, a reason suggested for the inability of other replicons to release infectious particles, for successful replication in vitro [162, 170]. In vitro transcribed full length JFH-1 genome or a recombinant chimeric genome with another genotype 2a isolate, secreted infectious virus particles in Huh7 and Huh7.5 cells [172-175]. These cell culture grown virus particles have a spherical shape, have 1.15-1.17g/ml density and are 55nm in diameter [172]. They were infectious in infectious in cultured cells, chimeric mice and chimpanzees [172-175]. The JFH-1 isolate allowed the complete life cycle of HCV to be studied giving greater insight into the underlying mechanisms compared with other available in vitro models [174-177].

1.1.5.1.4 HCV Pseudoparticles (HCVpp)
Another cell-based model system that contributed to study HCV viral binding and entry is HCVpps. HCVpps are defective lentiviral particles expressing HCV glycoproteins which are made by assembling HCV glycoproteins E1 and E2 onto retroviral or lentiviral core particles. The HCVpps can mimic the viral entry of HCV [5, 162]. A reporter gene is also packaged in these particles to allow identification of infected cells, thus creating a system to examine virus entry to the cell [178]. HCVpp enabled studies of the entry aspects of the virus life cycle and were used in determining the role of HCV entry factors like CLDN1 and CD81 [44, 178, 179]. They also provided functional assays to screen the effect of neutralising antibodies on virus entry [178].
1.1.5.2 Animal models to study HCV

1.1.5.2.1 Chimpanzees
Chimpanzees are the only non-human primates that can be infected with HCV and hence used to study HCV infection; they can be infected with isolates of the 6 epidemiologically important genotypes [180]. Insights into the characterization of the virus, virus-host interactions and immunopathogenesis were determined by experimental infection of chimpanzees [162] and thus the use of chimpanzees, which have more than 98% genetic similarity with humans has contributed to our understanding of HCV infection [5]. In fact, chimpanzees were used to isolate cDNA from the Non-A, Non-B viral hepatitis genome which finally led to the identification of the virus as HCV [181]. Chimpanzees infected with HCV display elevated aminotransferases and liver biopsies show necro-inflammatory changes after acute infection. However, chimpanzees differ from humans in that their course of infection is milder; persistently infected animals do not develop cirrhosis or fibrosis and only one chimpanzee has been reported to have developed HCV-related hepatocellular carcinoma [182].

Immunological studies in chimpanzees including cellular immune kinetics have been particularly useful [183]. Nascimbeni et al. reported that the pattern of the cellular immune response in the blood and liver correlated with virological outcomes in a study that re-challenged chimpanzees which had previously cleared infection [184]. As described in sections 1.1.6.1 and 1.1.6.2, drug and vaccine development studies also largely used chimpanzees [185-187]. Overall chimpanzees are the gold standard animal models for HCV studies, however, their use as a model is banned in most countries primarily due to ethical issues and secondly due to economic reasons [5, 188].

1.1.5.2.2 Tupaia
Tupaia belangeri is a tree shrew native to Southeast Asia. Primary tupaia hepatocytes were shown to be susceptible to serum- or plasma- derived HCV infection [182, 189, 190]. However, these animals are not widely used as a model due to the low and variable HCV infection rates [180].
One study followed tupaia for three years after infection with HCV-positive serum or viral particles reconstituted from full length cDNA. The tupaia developed acute HCV infection and over time they developed chronic hepatitis that worsened progressively. Serum from infected tupaia also infected naïve tupaia. This report indicated that tupaia can be potential model for HCV infection, however, intermittent viremia might be a problem [191]. Moreover, the difficulty to genetically manipulate the animals, and their genetic heterogeneity, as a result of absence of inbred strains of tupaia, represent other limitations with these animal models [182, 192, 193].

1.1.5.2.3 Mouse models for HCV

Mice are not naturally susceptible for HCV infection, but sophisticated genetic manipulations have been used to make them models for HCV infection and pathogenesis. As a result, they have been used with different approaches to bridge between in vitro and in vivo experiments [192, 194].

In 2001, severe combined immunodeficient (SCID) mice carrying the homozygous plasminogen activator transgene (Alb-uPA) with chimeric human livers were developed [195]. The Alb-uPA transgene targets urokinase over-production to the liver resulting in a profoundly hypofibrinogenemnic state and accelerated hepatocyte death facilitating the transplantation process [196]. It was reported that following transplantation with human liver more than 50% of the liver cross-section was populated with human hepatocytes. These mice showed prolonged HCV infection with high clinically-relevant titres, ranging from 3x10⁴ to 3x10⁶ copies/ml of viral RNA, up to 35 weeks after inoculation with human serum. They support HCV infection with genotypes 1a, 1b, 3a and 6a [195].

In 2012, Ilan et al. developed another mouse model, termed the Trimera Mouse model. This model was developed by transplantation of HCV-infected human hepatocytes to lethally irradiated mice reconstituted with bone marrow cells from SCID mice [197]. HCV RNA was
detected in 50% of the transplanted mice on day 12 following transplantation and viral persistence was detected for one month. Treatment of these mice with small molecule antiviral, I70, and anti-HCV MAb, HCV ABXTL 68, reduced the mean viral load in a dose dependent manner [197].

An additional model, the recombination activating gene 2 (Rag2) knockout mouse model was able to support HCV and HBV infection after transplantation with human hepatocytes. Up to 95% chimerism was reported in these models which were evaluated for antiviral drug testing. Consequently, antiviral treatment with pegylated (peg)-IFN alone or peg-IFN with ribavirin (RBV) or peg-IFN with Debio-025, a cyclophilin inhibitor that was shown to reduce HCV viral load in treatment naïve patients when used in combination with peg-INF [198], for two weeks resulted in a 3log_{10} reduction in HCV RNA after two weeks [199]. All of the aforementioned mice models support HCV infection but due to either an immunosuppression or a mismatch in the immune mechanisms they cannot be used in vaccine-related studies [200]. High sensitivity to handling conditions and limited access to fresh human hepatocytes for engraftment are also limitations of these models [201].

The presence of a normal immune system in mice theoretically reflects the natural immune response in humans. Hence immunocompetent mice are vital to study the efficacy of vaccines against HCV [202]. In 2011 an immune deficient Rag2 knockout mice was developed into an immunocompetent model after reconstituting the immune system with human hematopoietic stem cells. This model supported both HCV replication in the transplanted human liver and showed T-cell response against the virus. However, only 50% of the mice were infected with HCV and none of them developed HCV viremia [203].

Another immunocompetent model was developed by using a bicistronic HCV genome expressing CRE recombinase which activates a luciferase reporter molecule. Expression of the reporter signal indicates expression of HCV genome in the liver of the mouse. In this model adenovirus
(Ad) transduction was used to express the human cellular factors (CD81 and OCLN) necessary for HCV entry. All mice expressing CD81 and OCLN were successfully infected with HCV but there was a reduction in the signal 72 hours post infection, which may be related to anti-vector immunity [204]. The model was then modified to stably express all the human factors necessary for HCV entry. Similar to the previous model, reporter activation was observed following HCV CRE infection. Administration of antibodies against the HCV envelope or the host entry factor CD81 resulted in a dose-dependent inhibition of HCV infection. These observations suggested that the model can be used to assess the efficacy of vaccine strategies, at least those passively administered immunizations [205]. None of the above models was used in challenge experiments to examine HCV specific CMI.

The intra-hepatic challenge model developed in the Gowans’ laboratory in 2014, showed promising results to test the efficacy of vaccines for HCV [206]. In this model hydrodynamic injection of a HCV gene plus a secreted alkaline phosphatase (SEAP) reporter was used as a “challenge”. An effective immune response resulted in a decrease in serum SEAP levels. The co-expression of SEAP allowed a more sensitive method of detecting HCV gene expression in the mouse liver and also enabled longitudinal analysis of the immune response in mice without the need to sacrifice the animals [206]. However, this model has not yet developed in a large animal model.

An immunocompetent, HCV permissive mouse model with evidence of liver disease resulting from HCV infection was reported by Chen et al. [207]. The group reported the creation of a transgenic mouse in ICR (CD1) background (C/O^Tg) expressing human OCLN and CD81 that were permissive to infection with serum- and cell culture-derived HCV and maintained stable levels of HCV RNA in serum and in liver for over 12 months. In situ hybridization of HCV RNA and staining of HCV NS5A protein in the liver cells of mice at different time points confirmed
sustained infection. Moreover, a HCV protease inhibitor cured the infection. The production of infectious viruses was further confirmed by successful infection of Huh7.5.1 cells with mouse sera and successful passage of HCV into naïve C/O Tg mice [207].

1.1.6 Treatment and prevention of HCV infection

1.1.6.1 Treatment

Conventional IFN, recombinant IFN-α, was used as treatment for non-A, non-B hepatitis before the infectious agent was identified as HCV, but response to treatment was not encouraging as a sustained virologic response rate (SVR) was less than 20% [208]. Combination therapies with recombinant PegIFN and RBV, which was the standard therapy for a decade (2001-2011) improved the antiviral efficacy. This combination resulted in 40%-50% SVR in genotype 1 and up to 80% in genotypes 2 and 3 [209]. However, the SVR among treatment-experienced cases was lower compared with treatment-naïve patients [210]. A nationwide study from Germany showed that the SVR in treatment naïve patients was 54.1% while it was 43.5% in retreated cases [211].

The development of directly acting anti-virals (DAA) has changed the history of HCV treatment. Combinations of these specifically targeted drugs and PegIFN and RBV has resulted in an increased rate of SVR, while more recent DAA are PegIFN and RBV free. Based on their target site DAAs can be classified in to three major groups, viz NS3/4A protease inhibitors (PI), NS5A inhibitors and RNA-dependent NS5B polymerase inhibitors [212, 213].

**NS3/4A PI:** The NS3/4A is a complex bi-functional molecule essential for viral polyprotein processing and RNA replication [3]. The first generation of DAAs included boceprevir (BOC) and telaprevir (TVR) both of which are NS3/4A PI and were approved for use in combination with PegIFN α and RBV in 2011. First generation NS3/4A PIs are largely genotype 1 specific and have an inherent low barrier to viral resistance, but later versions have improved potency and
wider genotypic coverage [214]. A recent report from Sweden showed that triple therapy using a combination of PegIFN, RBV and either BOC or TVR resulted in SVR of 56% [215]. In Brazil the use of these drugs has been discontinued in the clinic due to their high cost and drug related adverse events [216].

Later in 2013 simeprevir (SMV) was approved as a second-generation PI to be used in triple therapy with PegIFN and RBV [168, 213]. In a study that assessed the prevalence and impact of baseline resistance-associated substitution (RAS) in treatment outcome, it has been reported that the presence of baseline SMV RAS was associated with a small reduction in SVR in HCV patients infected with gt1a but had no impact in gt1b infected patients [217].

Grazoprevir is another second-generation NS3/4A PI that demonstrated activity against RAS seen after failed therapy with first generation PI. This drug has been developed to be used in combination with the NS5A inhibitor, elbasvir, for the treatment of chronic HCV gt1 or gt4 infections [218, 219]. Oral elbasvir/grazoprevir 50/100 mg (Zepatier™) once daily has been approved by the United States Food and Drug Administration in 2016 [220].

**NS5A inhibitors:** The NS5A viral protein is essential for RNA replication and assembly of infectious virions via mechanisms that remain poorly understood [214]. Minimal inhibition of NS5A is associated with significant reductions in HCV RNA levels in cell culture-based models, which makes NS5A inhibitors among the most potent antiviral molecules yet developed [221]. NS5A inhibitors have pan-genotypic activity, i.e., they suppress replication of all HCV genotypes, but their antiviral effectiveness against different genotypes may vary between compounds [222]. Currently approved NS5A inhibitors are ledipasvir (LDV), daclatasvir (DCV), ombitasvir, elbasvir, velpatasvir (VEL) and pibrentasvir (PIB) [223].

Daclatasvir (DAC) was the first NS5A inhibitor to be developed and approved in 2015 for the treatment of HCV gt3 [224, 225]. Ombitasvir is another NS5A inhibitor with picomolar potency
and pan-genotypic activity [168, 225]. LDV was approved to be used in combination with the NS5B nucleotide analogue sofosbuvir (SOF) [226]. Elbasvir showed activity against gt1a, 1b, and 3 in vitro, including against some viral variants resistant to other NS5A inhibitors [227]. VEL was assessed for its efficacy in combination with SOF in a double-blinded, placebo controlled phase 3 trial (ASTRAL-1) and a combination of these two anti-viral drugs showed 99% (95% confidence interval, 98 to >99) SVR among patients infected with HCV gt1, 2, 4, 5 and 6 [228].

Likewise, PIB was tested in combination with glecaprevir, a NS3/4A PI, against HCV gt1-6 and results showed higher rates of SVR against these genotypes [229, 230].

**NS5B inhibitors:** NS5B is RDRP which is necessary for virus RNA replication. The enzyme has a catalytic site and four other sites which are targets for allosteric inhibition [231] and consequently drugs that target the NS5B polymerase are either nucleotide inhibitors (NI) or non-nucleotide inhibitors (NNI) [232]. The binding of these compounds to the NS5B polymerase inhibits conformational changes required for its polymerase activity [233].

SOF, a NI, is a uridine analogue that mimics natural cellular uridine and is incorporated by the HCV RNA polymerase into the elongating RNA strand, resulting in chain termination [168]. Given that the catalytic site of the NS5B protein is highly conserved across HCV genotypes 1–6, this mechanism is believed to account for the broad genotypic activity demonstrated by SOF in HCV replicon cells [234].

Dasabuvir (DVR) is a NNI that is able to mediate viral clearance, mainly HCV gt1, when used in combination with other antiviral agents [235, 236]. DVR is administered in combination with paritaprevir (NS3/4A PI)/ombitasvir (NS5A inhibitor) in the presence of a pharmacokinetic enhancer, ritonavir [237].

Although these DAA are effective, they may induce or select drug resistant mutants due to the high replication capacity of the virus and the low fidelity of the RDRP resulting in numerous
variants which are produced during HCV replication. These may include variants with altered binding sites for DAA that may be selected to become dominant variants. Thus, selection of resistant variants is an intrinsic feature of DAA [238].

Another major problem associated with DAAs is the cost of treatment [239]. According to a study published in 2016, across 26 countries in the Organisation for Economic Co-operation and Development (OECD), the median price for a 12 week course of SOF was US$ 42,017 [240]. A more recent report from the United Kingdom stated that a deal between manufacturers and NHS England has been secured to bring down the treatment cost to about £5000 [241]. When it comes to developing countries with resource limited settings, the use of these drugs is unaffordable which is also the case in several European countries (e.g. Belgium, The Netherlands, Spain, Italy), where DAAs can only be used in patients with advanced fibrosis or cirrhosis [17].

In addition to the limitations noted above, 80% of HCV infected individuals are unaware of their sero-status precluding them from treatment [7, 242, 243]. After lengthy treatment period of 8-24 weeks that involved high cost, treated individuals treated who showed a SVR remain susceptible to reinfection either from a new exposure or a relapse from previously treated HCV, which was observed in patients treated with IFN therapy [244-246].

Considering the global impact of HCV and the limitations associated with HCV treatment there is an urgent need for a vaccine. Vaccines strategies are of great public health importance as they are cost-effective and represent a realistic means to significantly reduce the worldwide mortality and morbidity associated with HCV infection [243].

1.1.6.2 Vaccines
Vaccination is an established method to generate immunity against specific pathogens. There are different methods, described below, to make vaccines [247].
1.1.6.2.1  Live attenuated vaccines (LAV)

LAV have played critical role in controlling many human diseases, among which small pox eradication is one of the successful medical stories [248]. An important goal in vaccine development is to avoid disease from the vaccine itself. To this end, attenuation of viral vaccines is achieved through repeated passaging of the wild type virus in cell cultures [249]. LAV have been successfully used to prevent infections by poliovirus, measles virus, rubella virus and yellow fever virus [248, 250]. Apart from the unpredictable method of attenuation, the main drawback of LAV is the potential of reversion to virulence [251]. Hence this technology is unsuitable for HCV as a consequence of the high mutation rate [252].

1.1.6.2.2  Inactivated virus vaccines

Using either chemical or physical methods, viruses can be inactivated so they will no longer revert to an infectious particle while they are recognized by the immune system [253]. Although inactivated vaccines are safer than LAV, they are weaker in eliciting immune response and hence may require adjuvants and booster dose [248, 254]. Examples of inactivated virus vaccines include vaccines against hepatitis A virus and polio virus [255].

Purified, inactivated HCVcc derived from the J6/JFH-1 chimeric genome, was used to immunize mice [256]. Sera collected from vaccinated mice contained antibodies against the HCV envelope that inhibited infection of cultured cells with HCV genotypes 1a, 1b, and 2a. Moreover, passive transfer of IgG from the immunized mice into uPA-SCID mice with humanized livers prevented infection with the minimum infectious dose of HCV [256]. However regulatory issues and low yield of HCV particle production in cell culture may limit the use of these vaccines [257, 258]

1.1.6.2.3  Subunit vaccines

Subunit vaccines are produced using only part of the virus to raise a protective immune response and because these vaccines cannot replicate in the host, there is no risk of pathogenicity [259]. Immune responses generated as a result of subunit vaccine are typically weak requiring the
incorporation of strong immune-stimulatory components and booster doses [260]. The inactivated subunit influenza virus vaccine and HBV vaccine are licensed vaccines under this technology [261, 262].

As far as HCV is concerned, a recent report showed that soluble HCV E2 (sE2) protein produced in insect cells induced immune responses that prevented infection in a genetically humanised mouse model [263]. Further immunogenicity characterisation in macaques revealed that the vaccine was able to elicit NAbs against HCVcc harbouring structural proteins from multiple genotypes [264].

1.1.6.2.4 Virus like particles (VLP)

VLP are multi-protein supra-molecular structures that mimic the virus particle but do not contain viral genome. VLP-based vaccines are generally considered as safe because they do not replicate [265]. VLPs provide polyvalent structures that can accommodate multiple copies of antigens and are able to stimulate immune cells [266]. The HPV vaccine is an example of a VLP vaccine [266].

Garrone et al. reported that VLPs pseudotyped with HCV E2 and/or E1 induced high-titre anti-E2 and/or anti-E1 antibodies, as well as NAbs, in mice and macaques. The NAbs, which were raised against HCV 1a, cross-neutralized five other genotypes tested (1b, 2a, 2b, 4, and 5) [267]. More recently, it has been reported that a quadrivalent gt1a/1b/2a/3a VLP vaccine was able to induce high titre antibodies when adjuvanted with a TLR 2 agonist, although the adjuvant did not change the strength of the NAbs [268]. Further investigations showed that the quadrivalent VLP vaccines was able to induce strong T cell immunity as determined by IFN- γ Enzyme-linked immunosorbent spot (ELISpot) assay [269].
1.1.6.3 HCV vaccines

Vaccines against HCV need not to be protective against infection; rather, prevention of viral persistence will prevent the bulk of HCV associated disease [247]. In the next sections the different approaches used to develop vaccine against HCV are discussed.

1.1.6.3.1 Vaccine approaches to elicit NAb

Studies in small animal models and non-human primates [270-272] indicated that NAb are important in the prevention of HCV persistence. However the heterogeneity of HCV limits the ability of NAb to be cross protective [257], although cross NAb that recognize conserved regions within E2 have been described [273, 274]. The following text reports the results of a few representative studies.

The use of envelope glycoproteins E1/E2 in vaccine formulations is one of the strategies used to induce NAb against HCV [257]. A meta-analysis of vaccine efficacy based on chimpanzee models suggested that NAb can play role in protection against HCV. According to the meta-analysis, vaccines with the highest protective efficacy included all or part of the envelope proteins inducing neutralizing antibody, E1E2 T-cell responses or both [185].

Five of seven chimpanzees vaccinated with HCV-1 purified recombinant E1E2 heterodimer in combination with MF59 were protected from IV challenge with homologous virus [275]. Later this vaccine was tested in a heterologous challenge after chimpanzees were reimmunized with recombinant E1E2 [276]. Following vaccination a strong and persistent NAb response was observed although chimpanzees developed acute HCV infection following challenge [276]. Similarly, a vaccine made from the combination of recombinant E1 and E2 from strain HCV-N2 and HRV 1 from isolate HCV-#6, induced high humoral immune response against E1 and E2 and low immune response against HRV1, was reported as non-protective against heterologous challenge with HCV-#6. However, protection was achieved when the anti-HRV 1 titre was increased suggesting that recombinant E1 and E2 vaccination alone is not protective [277].
A DNA vaccine encoding HCV E2 elicited anti-E2 responses but was unable to induce sterilising immunity in chimpanzees after three doses of 2mg of DNA, because both vaccinated and unvaccinated chimpanzees developed infection following homologous challenge. However, vaccinated chimpanzees were able to clear the infection in a shorter time while the unvaccinated chimpanzees developed persistent infection suggesting that the vaccine has modified the infection after challenge and prevented development of persistent infection [278].

Some of the vaccines that contain viral glycoproteins were tested in human clinical trials. A report by Law et al. showed that a vaccine comprising E1/E2, adjuvanted with MF59C.1, derived from a single isolate (gt1a) induced broad NAb against all seven major clades [279]. Volunteers were vaccinated intramuscularly with recombinant E1E2/MF59C.1 at 0, 4, 24 and 48 weeks and antibodies were detected by ELISA. [280]. The neutralising ability of these antibodies was shown by their ability to prevent chimeric HCVcc infection of Huh 7.5 cells in vitro [279].

Another phase I clinical trial to assess safety and immunogenicity of a recombinant E1 protein vaccine based on vaccinia virus adjuvanted with alum reported that the vaccine was tolerated and had no adverse effects [281]. Immunological assessment showed that all 20 individuals who received three doses of the vaccine plus a booster dose seroconverted and had higher level of antibody than the levels measured 2 weeks after the third dose although the neutralisation capability of these antibodies was not reported [281].

1.1.6.3.2 Vaccine approaches to elicit CMI
There are different approaches to make vaccines that can induce CMI. The use of synthetic peptides is a simple approach to develop a HCV vaccines [247]. IC41, a synthetic peptide vaccine candidate, was shown to be safe in a randomized, placebo-controlled trial among 128 individuals. This vaccine comprised poly L-arginine as a synthetic adjuvant and five synthetic
peptides that harbor HCV T cell epitopes [282]. IFN-γ ELISpot assay showed CD4⁺ and CD8⁺ T cell responses in 50% and 40% of the vaccinated individuals, respectively [282].

An Ad-based vaccine against HCV was shown to induce sustained T cell response in humans. HCV gt1b NS proteins encoded by human Ad6 and chimpanzee adenovirus 3 (ChAd3) were used to vaccinate volunteers and the vaccines were shown to be safe [283]. Immunologically, both vaccines primed T cells that targeted 5 peptide pools, though the NS3 peptide pool was the immunodominant, as assessed by IFN-γ ELISpot [283]. Functional analysis of the antiviral response by intracellular cytokine staining (ICS) showed that both CD4⁺ and CD8⁺ T cell subsets secreted interleukin-2, IFN-γ and TNF-α. The responses were sustained for at least one year after boosting with the heterologous Ad vector [283]. The response to heterologous strains of HCV showed 50% and 80% lower response to HCV gt1a and HCV gt3a, respectively, as compared to the priming gt1b [283]. Heterologous boosting, ChAd3 followed by ChAd6 or vice versa, failed to result in a significant increase in the magnitude of the immune response and this was speculated to be related to an anti-vector immune response. To overcome this problem the vaccination strategy was later modified by using Modified Virus Ankara (MVA) encoding NS3, NS4, NS5A and NS5B as boosting vector following vaccination with ChAd 3 [284]. This approach resulted in enhanced HCV-specific T cell response with extended duration, greater breadth and poly-functionality [284].

Another approach towards vaccine formulation to induce CMI is the use of DNA vaccines that involve the production of non-viable, non-replicating, non-spreading antigens that can induce CD4⁺, CD8⁺ T-cell immunity and B-cell immunity. Furthermore, DNA vaccines are safe as there is no risk of reversion because microorganisms are not directly used [285].

A recent report indicated that a DNA vaccine that encoded the HCV NS3/4/5B polyprotein from gt3a and a cytolytic protein, perforin (PRF), generated HCV-specific IFN-γ responses in mice
Another cytolytic DNA vaccine encoding HCV NS3 from gt1b was also shown to induce robust CMI in mice and pigs; the immune response was characterized by significantly higher levels of IFN-γ response and NS3 specific CD8+ T cells, although statistical significance in the pig study was not shown due to the small number of pigs [287].

A universal vaccine that can induce CMI against the different genotypes may be necessary to eradicate HCV. To this end, Wijesundara et al. tested the immunogenicity of a cytolytic DNA vaccine cocktail encoding NS3, 4A and 5B from HCV gt1a and gt3a [288]. According to this study, in vivo T cell responses, as determined by fluorescent target array (FTA), were clearly observed for gt1b and gt3a NS3 and NS5B [288]. Using a combination of NS3/4A, NS4B, NS5A and NS5B in a single vaccine formulation, others also showed that DNA-based HCV vaccines are strongly immunogenic and can induce broad T-cell reactivity in macaques, characterized by high numbers of HCV specific CD4+ and CD8+ T cells that were detectable 6 months post vaccination [242]. Others also reported that DNA vaccines that encode individual non-structural genes are able to induce cellular immunity [289-291].

A DNA vaccine encoding HCV NS3/4A was reported as being safe and well tolerated among individuals chronically infected with HCV [292]. Vaccinated patients showed increased IFN-γ production during the first 6 weeks. Six of eight patients who were under standard of care treatment with PegIFN and RBV showed SVR 1-30 months after vaccination [292].

A recombinant pox virus vaccine (TG-4040) encoding HCV NS3, NS4 and NS5B was able to induce HCV-specific CMI and reduced the viral load in chronic HCV patients [293]. CIGB-230 is a therapeutic DNA vaccine candidate that contains plasmid-encoded HCV core, E1 and E2 polyprotein from HCV genotype 1, a recombinant HCV core protein and pIDKE2 (a plasmid encoding the first 650 amino acids of the HCV polyprotein) [294, 295]. In a phase I clinical trial this vaccine was able to induce a specific T cell proliferative response and a T-cell IFN-γ secretory
response in 73% of the study subjects. Moreover, despite persistent detection of HCV RNA, more than 40% of vaccinated individuals improved or stabilized liver histology, particularly reducing fibrosis [294].

Despite the use of different approaches for the development of vaccines for HCV, there is no licensed vaccine so far. A major obstacle of vaccine development is the genetic diversity of the virus as a result of the error prone RDRP [243] that enables the virus to escape from vaccine induced immune response [296]. The lack of appropriate model systems, ranging from cell culture to animal models, is another factor that impedes the development of an effective vaccine for HCV [252].

1.2 Adeno associated virus (AAV)

1.2.1 Virus structure and genome organization
The adeno-associated virus (AAV) was discovered in 1965 as a contaminant of simian Ad preparations [297]. AAV requires a helper virus for a productive infection and was classified as a Dependovirus in the Parvoviridae family [298]. The AAV virion is an icosahedral non-enveloped particle which measures 20-25 nm [299, 300].

AAV serotypes have been isolated from different species including primates, avian and bovine. AAV serotypes 2, 3, 5, and 6 were isolated from human cells, while AAV serotypes 1, 4, and 7–11 have nonhuman primate origins [299]. The general organization of the parvovirus genome is conserved across the different serotypes, with a similar configuration of replication and structural genes [299]. More than 110 primate AAV capsid sequences have been isolated and classified under 12 serotypes [301]. Based on the prevalence of NAb AAV2, 3, and 5 are thought to be of human origin while AAV4 originated from non-human primates [302].

The genome of AAV is a 4.7kb ssDNA that has three open reading frames (ORF), rep, cap and aap (Figure 1.4). The ORFs are enclosed between two inverted terminal repeats (ITR), which are
Cis-acting components of the viral genome that function as the viral origin of replication and packaging signal [303-305]. The gene contains three viral promoters that are identified by their relative map position within the viral genome: p5, p19, and p40 [299]. Each promoter controls the expression of different proteins [306].

![Diagram of AAV genome](image)

**Figure 1.4: The genome of AAV.** The AAV genome has three ORFs flanked by ITRs (blue). The rep ORF (red) codes for four Rep proteins (Rep78, Rep68, Rep52, and Rep40) that are synthesized from mRNAs initiated from the p5 and p19 promoters. The cap ORF (yellow) codes for the three capsid proteins (VP1, VP2 and VP3) transcribed from mRNA controlled by p40. The VP2/VP3 mRNA codes for an assembly-activating protein (AAP) (green) AAV [300].

### 1.2.1.1 Cap gene

Transcription of the cap gene controlled by the p40 promoter leads to the production of VP1, VP2 and VP3 (Figure 1.4) [298]. Together these proteins form the viral capsid which is composed of 60 subunits arranged in an icosahedral symmetry [307]. The different structural proteins are produced following alternate messenger RNA (mRNA) splicing [308]. VP1 is translated from the minor spliced mRNA while VP2 and VP3 are both translated from the more abundant major spliced mRNA [307, 309].
The first step of AAV infection is attachment of the virus to the host cell. This important step is mediated by structures on the capsid of the virus which interact with the cellular receptors found on specific cells (section 1.2.2.1), resulting in the range of tissue tropism seen for the various AAV serotypes [307, 310, 311]. The capsid also determines the transduction activity of rAAV in different organs [312].

VP1, VP2 and VP3 are found in a ratio of 1:1:10 in the capsid. The ratio of VP3 is 10x higher than VP1 and VP2 because VP1 is translated from minor spliced mRNA while VP2 translation is initiated from a less efficient ACG codon, although it is translated from major a spliced mRNA [307]. The N-terminus region of VP1 has a unique phospholipase A2 (PLA2) motif and a putative nuclear localization signal. The phospholipase domain is proposed to play role in virion escape from the endosome. Mutations in the VP1 were shown to decrease infection and impair trafficking suggesting the involvement of this protein in intracellular trafficking of the virus [310, 313].

Serology and receptor usage of AAV is determined by the VP3 which consists of a conserved β-barrel motif of anti-parallel β-sheet [299]. There are controversies around the role of VP2 in virus assembly and packaging. Previous reports state that VP2 is necessary for particle formation in SF9 cells [314]. According to this study, capsid assembly using the three proteins expressed from separate constructs in the absence of viral DNA or helper virus suggested that VP1 plus VP3, VP1 plus VP2, or VP2 alone can form virus-like particles [314]. A similar study with HeLa cells suggested that VP1 or VP2 alone, but not VP3, can form intact particles [315]. This is contrary to a study which reported that the VP2 protein is not essential for assembly or infection [316]. Another study reported that VP2 was not necessary for virus packaging as demonstrated by packaging of rAAV in the absence of VP2. rAAV packaged from two VP was as efficient in transducing cells as the rAAV packaged from all three VP [317].
1.2.1.2 Rep gene

The rep ORF encodes the four NS proteins that are designated by their molecular weight as Rep78, Rep68, Rep52 and Rep40. Transcription of Rep 78 and Rep 68 mRNA is facilitated by the p5 promoter while transcription of Rep52 and Rep40 is mediated by the p19 promoter. Rep78 and Rep68 are involved in the various stages of the AAV life cycle including DNA replication, site-specific DNA integration (section 1.2.3) and regulation of gene expression [310] and have helicase-, strand- and site-specific endonuclease- activities [318, 319]. The Rep52 and Rep40 proteins are DNA helicases essential for the accumulation and packaging of the genome into the capsid [320].

1.2.2 Replication of AAV

1.2.2.1 Viral entry and trafficking

Attachment of AAV to the host cell involves an interaction between the viral proteins and primary- and co-receptors. At least for the best studied serotype, AAV-2, the primary receptor is HSPG determined by a competition assay and mutant cell transduction assay [301].

AAV2 binds directly to cell surface HSPGs via an HS-binding motif on the virus capsid. Cultured hepatocytes from mice which lack the EXT1 gene, which encodes EXT, an enzyme which is involved in the biosynthesis of HSPG, showed low level of transduction with AAV-2 [321]. Immunohistochemical analysis of liver sections from EXT 1 mutant mice also showed low level of expression of the transgene, indicating that HSPG is important for in vivo transduction of mouse liver [321]. Tissue tropism of AAV-2 in a broad range of hosts may be associated with the use of HSPG as the primary attachment receptor [298].

For successful viral entry HSPG-mediated attachment of AAV to the host cell is aided by co-receptors like the hepatocyte growth factor receptor and integrins [301]. C-Met, a receptor for hepatocyte growth factor which is expressed both on epithelial and non-epithelial cells acts as a
co-receptor for AAV-2. Cells constitutively expressing c-Met showed an increased level of GFP expression after transduction with AAV-GFP as compared to cells lacking the protein. However, experiments that assessed the level of attachment failed to show any difference between c-Met expressing and non-expressing cells, but there was increased rate of internalization of AAV to c-Met expressing cells [322].

Integrins also participate in the internalization of AAV 2. The role of ανβ5 integrins, molecules involved in cell adhesion and motility was studied by Sanlioglu et al. [323]. According to this study antibody-mediated blockage of these integrins inhibited the uptake of AAV2 bound to HSPGs [323]. In another study chemical disruption of integrins using ethylene diamine tetraacetic acid (EDTA)-mediated chelation resulted in a reduced level of infection with AAV, indicating that integrins are also important co-receptor in the infectious process of AAV [324].

The laminin receptor (LamR) is the first receptor described to play role in AAV-8 binding and transduction [325, 326]. Antibody mediated inhibition of LamR or laminin mediated blockage of LamR on HeLa cells significantly inhibited AAV-8 binding to the cells while overexpression of LamR on NIH 3T3 cells resulted in increased transgene expression following transduction with GFP encoding AAV-8 [325].

When it comes to other AAV serotypes sialic acid has been shown to be important in binding and transduction for serotypes 1, 4, 5, and 6 [298, 301]. Binding and transduction studies by including O-linked and N-linked sialic acid glycosylation inhibitors revealed that AAV-4 binding required O-linked sialic acid while AAV-5 needs N-linked sialic acid [327]. Similarly, for AAV-1 and AAV-6, genetic and enzymatic removal of sialic acid markedly reduced binding and transduction of HepG2, Pro-5 and Cos-7 cells by these serotypes. Moreover, proteinase K treatment of cells, but not glycolipid inhibitors, reduced infection of cells by these serotypes suggesting that sialic acid is important for binding of AAV-1 and AAV-6 [298, 301, 328, 329].
More recently, a universal receptor for most of the AAV serotypes has been described by Pilly et al. [330]. The receptor is a type I transmembrane protein, KIAA0319L, and was named AAV receptor (AAVR). The authors reported that AA2VR was essential for AAV transduction of human cells derived from a broad range of tissues and in an in vivo mouse model [330, 331].

Following attachment to the host cell, successful transduction by AAV requires the virion to progress through internalization, cytoplasmic trafficking and finally nuclear entry [332]. AAV is internalized through receptor-mediated endocytosis via clathrin-coated pits or through the clathrin-independent carriers/glycophosphatidylinositol-enriched early endosomal compartments (CLIC/GEEC) pathway [333]. To study the role of dynamin (a cytosolic GTPase), involved in clathrin-dependant endocytosis [334], Stoneham and colleagues used AAV/phage (AAVP) which was generated as a chimera between AAV and a derivative of filamentous M13 bacteriophage [335]. HEK 293 T cells were treated with dynasore prior to- or during- incubation with AAVP. Flow cytometric analysis of transduced cells following inhibition of dynamin activity with dynasore resulted in poor uptake of AAVP as compared to the dimethyl sulfoxide (DMSO)-treated control which had little or no effect on endocytosis. This was also observed when cells were transduced in the presence of a plasmid expressing mutant dynamin II-K44A tagged with GFP. A 57% reduction in GFP expressing cells was noted as compared to the non-transfected cells [335].

Following virus internalisation, the viral genome has to be processed by the host cell for successful gene expression from AAV genome. Gene expression from AAV vectors is characterised by delayed detection. Different reasons are proposed for this; the first one is since the AAV genome is ssDNA, the slow conversion of ssDNA to dsDNA is the primary cause for the delayed expression profile [336, 337]. Intracellular trafficking is the second reason suggested to be a determinant factor in the AAV mediated gene expression [332, 338]. From the studies that
support this hypothesis, Hansen et al. claimed that gene expression in NIH 3T3 cells following AAV transduction was not successful due to impaired intracellular trafficking, although these cells have all the necessary receptors and co-receptors required for viral attachment and entry [338]. To show this they performed Southern blot analysis of DNA isolated from AAV infected NIH 3T3 and 293 cells at different time point after infection. Accordingly, within 2 hours, ~76% of the input ssDNA was present in the nucleus of 293 cells while in NIH 3T3 cells essentially all (~99%) of the signal was detected in the cytoplasm. At 48 hours post infection the majority (~82%) of the signal remained in the cytoplasm of NIH 3T3 cells suggesting that AAV vectors attached and internalized successfully but trafficking to the nucleus was impaired [338].

Among the steps in the intracellular trafficking of AAV, endosomal processing represents an important step in the infectious process of the virus [339]. Once in early endosomes, viral particles can be further transported to the late endosomal compartment or released in the cytosol. AAV have been shown to undergo late endosomal processing and utilize microtubules to traffic its genome into the nucleus of infected cells [332].

Douar et al. 2001 used brefeldin A to test if AAV is processed further than the early endocytic vesicle [340]. Brefeldin A is a compound that prevents protein transport from the Golgi compartment to the ER and prevent early-to-late endosome transition [340, 341]. According to this study, treatment with brefeldin A led to a decrease of 2 to 3 orders of magnitude in the efficiency of gene transfer, as measured by the level of luciferase activity in 293, HepG2 and HeLa cells transduced by rAAV encoding luciferase (rAAV-Luc) [340].

In another similar study, impaired endosomal processing in NIH 3T3 cells was suggested to be the main reason for the absence of gene expression in these cell types [342]. In the more permissive 293 cells, virions were found in both early and late endosomes or lysosomes, while in NIH 3T3 cells virions were localised only to the early endosome [342]. This was evidenced by
the inhibition of endosomal-lysosomal acidification that decreased transduction of 293 cells while had no effect on NIH 3T3 cells. On the other hand, when NIH 3T3 cells were exposed to hydroxyurea, a compound known to increase AAV-mediated transduction, virions were detected in late endosome of these cells [342].

**1.2.2 DNA replication, gene expression and virus assembly**

AAV DNA replication is initiated by self-priming of the 3’ ITR using the host DNA polymerase (pol) δ. The genome is replicated through the 5’ ITR to produce a dsDNA intermediate (Figure 1.5A) that is usually closed at one end, termed turnaround form [343]. While copying the 5’ ITR a new 3’ ITR is generated and serves as a primer for the synthesis of a second strand [300, 304]. Simultaneously, the AAV Rep proteins (Rep78 and Rep68), bind to the Rep-binding element (RBE) on the original 3’ ITR, and cuts the lower strand at the terminal resolution site (trs) (Figures 1. 5 B and C) [344]. This process generates a free 3’ end (Figure 1.5 D), which allows the replication of the original 3’ ITR and the production of a new ssDNA genome as well as another dsDNA intermediate (Figure 1.5 E) which is fed back into the replication cascade [304].

In the case of unsuccessful nicking and terminal resolution (Figure 1.5 F and G) a self-complementary genome that serves as an intermolecular hinge will be formed. This hinge acts to form a duplex genome (dimer-length replicative form of DNA) (Figure 5 H and I) immediately upon uncoating and bypasses the typical need for second-strand synthesis that serves as a bottleneck for ssDNA vectors [304].

Because the two ends are identical, the process occurs equally well from both ends, generating both positive and negative strands for packaging [300, 345]. This replication mechanism results in the production of ssDNA genomes of both polarities, which are then packaged into the capsid [304].

The commonly accepted concept of AAV virion assembly suggests that empty capsids are formed into which single stranded DNA genomes are then introduced [346]. Assembly of the capsid is
facilitated by a protein called AAP. AAP is localized in the nucleolus, where AAV capsid morphogenesis occurs, and transports nascent proteins, mainly VP3 [346]. AAP from AAV-2 was shown to contain nuclear- and nucleolar- localization signals near its C-terminus region. These signals are responsible for the intracellular localization of AAP and are critical to its function in capsid assembly [346, 347].

1.2.2.3 Helper viruses and cellular proteins required for AAV replication

Productive replication of AAV requires helper functions from other viruses mainly Ad or herpes simplex virus (HSV) [298]. Ad helper functions are provided by the E1a, E1b, E4 orf6 and viral associated (VA) RNA. Both E1a and E1b act as transcriptional activators that induce the AAV p5 promoter. Chang et al. fused the chloramphenicol acetyltransferase (CAT) gene to a plasmid that contained the AAV p5 transcriptional control region to generate AAVp5-CAT. Transfection of HeLa cells with AAVp5-CAT in the presence of Ad E1a increased CAT expression by 7-fold while transfection in the presence of E1a and E1b proteins caused a 23-fold increase in CAT expression. The increased transcriptional activity of p5 in the presence of E1 is mediated by two elements; the Ad major late transcription factor (MLTF) binding site and a tandemly repeated 10-base pair sequence. These two elements interact to make p5 more responsive to E1a [348]. On the other hand, in the absence of Ad proteins Rep proteins produced from the p5 promoter autorepress the p5 promoter, producing barely detectable levels of Rep protein ensuring AAV genome latency [349]. E1b, E4 orf6, and VA RNA, perform various tasks that include promoting second-strand synthesis of AAV DNA [336], promoting AAV mRNA transport to the cytoplasm, and inhibiting the IFN-induced dsRNA–activated PKR (DAI/PKR) [300]. The product of E2b is a DNA binding protein that is found in the AAV replication complex and enhances in vitro replication of AAV DNA [299, 350].
Figure 1.5: AAV genome replication: AAV virions contain a linear ssDNA that replicate through a double strand intermediate turnaround form (A). Turnaround ends are nicked by Rep78 or -68 (closed circle), which becomes covalently attached to the 5’ end of the DNA (B). DNA polymerase extends through the terminal repeat to generate replicative form DNA that has two extended ends (C). Steps B and C are often referred to as terminal resolution. Isomerization of the terminal repeat generates a new hairpin, which serves as the primer for DNA synthesis (D) that allows the production of new ssDNA genome and another dsDNA (E) that feeds back to the replication cascade. If DNA synthesis begins prior to resolution of the turnaround end (F and G), the replication complex will proceed through the turnaround end and generate dimer-length replicative-form DNA (H and I).
The helper function of HSV seems to be important during DNA replication unlike that of Ad helper function which mainly is involved in gene regulation. Co-transfection of AAV2 with different HSV genes demonstrated that the HSV helicase primase multienzyme complex composed of UL5, UL8, UL52 and UL29 (major DNA binding protein) were sufficient to mediate helper function [351]. In contrast, by introducing a mutation in UL52 others demonstrated that the primase activity of UL52 is not required for AAV DNA replication as the mutated UL52 had no effect on AAV replication while the UL5 helicase activity was still essential for DNA replication [352].

Cellular factors that that support AAV DNA replication even in the absence of Ad-infection have also been described using an in vitro AAV DNA replication assay [353]. The standard in vitro AAV DNA replication assay contains linear AAV DNA, HeLa cell extract and Rep78 collected from crude extract of cells infected with a Rep78-expressing baculovirus [354]. In this model it was indicated that cell extract from Ad-infected HeLa cells supported in vitro replication of AAV DNA [354]. Later, it was shown that Ad-uninfected cells were able to support AAV DNA replication in vitro [353]. Addition of MAb or chemical inhibitors to the standard in vitro AAV DNA replication assay, helped to identify cellular factors involved in the in vitro AAV DNA replication [353]. Accordingly, antibodies against replication protein A (RPA) and replication factor C (RFC) caused significant inhibition of in vitro AAV DNA replication. The addition of aphidicolin, a chemical inhibitor of DNA Pol α, Pol δ and Pol ε, abolished DNA synthesis indicating either one or more of these polymerases is involved in DNA replication [353]. More recent data, based on fractionation assays of extracts from Ad-infected cells, suggested that Pol δ is the only active polymerase required for the replication of AAV DNA [355]. An additional cellular element required for in vitro reconstitution of AAV DNA replication was suggested to be minichromosome maintenance complex protein (MCM), which is likely to be a eukaryotic
replicative helicase [356]. This was shown by antibody-mediated blockage of MCM which resulted in the inhibition of AAV DNA replication. Moreover the addition of MCM to a replication assay system that contained purified Rep, proliferating cell nuclear antigen (PCNA), RFC and Pol δ was able to induce AAV DNA replication in vitro [356].

1.2.3 Site specific integration of AAV to the human genome
As a result of its defective nature, the AAV will enter a lysogenic pathway if no helper functions are provided by Ad or HSV. This makes AAV the only eukaryotic virus that has a property of establishing latency by integrating its genome into a specific site in the human chromosome 19q13.3-qter [357]. This event shows no effect on expression from the target locus. According to a study by Henckaerts and colleagues, integration of AAV to its target site will cause partial duplication of the target gene, but gene expression was not affected despite these rearrangements [358].

The minimum requirements for site specific integration of AAV DNA are the presence of the large Rep proteins 68 or 78, the viral Rep binding sequence (RBS sequence) and a cellular sequence, present in the AAV integration site 1 (AAVS1), consisting of a trs and RBS signal [344]. AAVS1 was shown to contain a repeated tetra nucleotide sequence of ‘GCTC’ which is similar to the sequence found in the ITR of the viral DNA. This site was identified as a binding site for Rep78/Rep68 proteins on the viral DNA suggesting that these regulatory proteins are involved in the process of integration [359]. Experiments to assess binding of Rep to the AAVS1 site showed binding of Rep78/Rep68 protein to a 109bp sequence in the AAVS1 site. Further analysis to identify the RBS showed that guanine residues in the GCTC motif was an important binding site [360]. Functional analysis of the AAVS1 sequence showed that a target sequence of 33 nucleotides containing the RBS and trs sequences is necessary and sufficient for AAV site-specific integration [361].
In a study that used transgenic mice carrying the human AAVS1, a 16bp RBE was shown to mediate Rep-dependent integration of the human coagulation factor IX (hFIX) gene. Transgenic mice were hydrodynamically injected with a hFIX expression plasmid (pRBE-CMV-hFIX) containing the 16bp RBE element in the presence of the Rep donor plasmid. Sequence analysis showed site-specific insertion in 4 of 7 animals, but no integration in animals infused with plasmids lacking the 16bp RBE (pN2-CMV-hFIX) or pRBE-CMV-hFIX plasmid alone. As a result, significantly higher levels of hFIX were observed in mice infected with pRBE-CMV-hFIX in the presence of Rep encoding plasmid [362]. The results from this experiment suggested that AAV site specific integration is both RBE- and Rep- dependent [362]. Others also demonstrated that this 16bp RBE is sufficient to mediate Rep-dependent integration into AAVS1 [363].

1.2.4 AAV serotypes and human infection

Approximately 80% of the population is seropositive for anti-AAV antibodies against serotypes 1, 2, 3, and 5. However, there is no observable pathology associated with AAV infection [299]. NAb against the different serotypes of AAV have been reported among different population groups including healthy children, adults and patients with different diseases [364-368]. A limitation to draw conclusion from these studies is that they used different antibody titres to define positive and negative.

A study which examined NAb against AAV-2, AAV-5 and AAV-8 among children with haemophilia reported a prevalence 43.5%, 22.8% and 22.6% respectively [364]. The prevalence of these antibodies showed an increasing trend from early childhood to adolescence [365]. A study which examined 888 samples from four different continents showed that anti-AAV2 seroprevalence ranging from 30% in the US to 60% in Africa. Low antibody titres were observed against AAV-7 and AAV-8 [366]. Another study of a Chinese population reported 92%, 89%, and 69% prevalence of NAb against AAV-2, AAV-3 and AAV-8 respectively [367]. Different groups from around the world reported 59.5% prevalence of NAb against AAV1 among
individuals with heart failure [368] and 37% prevalence of NAb against AAV-5 from HIV positive individuals [369].

1.2.5 Recombinant AAV (rAAV) as a viral vector: Developments and its features

The development of rAAV as a viral vector has resulted from its characteristics such as non-pathogenicity, low immunogenicity and ability to transduce both dividing and non-dividing cells [370, 371]. In addition, the small gene reduces the risk of recombination making it safe for human clinical trials. Moreover, AAV vectors have a broad host and cell tropism range and can transduce in vitro and in vivo [372].

Following the cloning of a complete AAV-2 genome it was realized that the three ORF are not cis-acting genome components for viral replication. This observation led to the suggestion that a transgene could be placed between the ITRs for the production of rAAV provided cap, rep and aap were supplied in trans making AAV an important viral vector [304]. The basic design of an AAV vector is relatively simple, in that it consists of an appropriately sized expression cassette flanked by ITRs, which mediate the replication and packaging of the recombinant vector genome by the AAV replication protein Rep and associated factors in producer cells [299].

AAV vectors are feasible and safe vectors in gene-directed therapy. As a result, they have been used to treat diseases like alpha anti-trypsin deficiency, phenylketonuria and neurological disorders [373-376]. The use of rAAV mediated gene therapy has also been examined in the treatment of haemophilia [377]. Phase I/II clinical trials conducted so far showed promising results towards getting treatment for missing coagulation factors; factor VIII (FVIII) for haemophilia A and factor IX (FIX) for haemophilia B [378-380]. Reports from mouse model studies indicated that rAAV encoding sequences of different antigens were able to transduce hepatocytes [381-383].
Although rAAV vectors are gaining popularity in studies involving the introduction of a new gene, as mentioned above, there are also difficulties in the use of these vectors. The first limitation was related to their production, as the use of Ad helper in the production process led to contamination of the AAV vectors, but the replacement of the helper functions with plasmids that encode the necessary helper genes eliminated the problem [304].

A second major limitation is associated with their transgene packaging capacity; rAAV can accept ~4.5 kb of foreign DNA, a vital point if the coding sequence of a therapeutic gene is larger than the packaging capacity [384]. Despite this, studies showed rAAV with inserts ranging from 5.2 kb to 8.9 kb; including a study from, Grieger and Samulski which concluded that serotypes from 1 to 5 can successfully package DNA ranging from 4.4 to 6.0 kb as shown by Southern blotting [317]. A similar study indicated that rAAV packaged an insert of 8.9 kb using a capsid from AAV 5 [385]. In contrast, Wu et al. reported that the packaging capacity of AAV cannot exceed 5.2 kb [386]. According to this study, attempts to package 8.6 kb insert size in AAV 2, 5 and 8 resulted in vector genomes that were heterogenous in length and none generated intact 8.6 kb vector genomes. Dot-blot hybridization using DNA isolated from the vector showed no signal after 5.2 kb size revealing that the packaged genome was truncated although the absence 5’ ITR was not checked. However, in vitro transgene expression from rAAV produced from larger insert size (>5.2 kb) was observed when HEK293 cells were transduced at high multiplicity of infection (MOI), though the mechanism was not fully understood [386]. One common theme to these studies is that as the genome size increases the transduction efficacy decreases.

One proposed way out to the low level of transduction is the use of self-complementary AAV (scAAV) vectors that is designed as a ss inverted repeat, which folds back upon itself to form a ds genome when entering into infected cells [387]. This strategy is mainly important to bypass the second strand DNA synthesis which is mentioned as a rate limiting step in AAV transduction (Section 2.3.1). McCarty et al. reported that the scAAV resulted a 5 to 140-fold increase in
transduction of HeLa cells and resulted significant increase in transgene expression \textit{in vivo} when compared to the conventional rAAV [388]. Others also reported more efficient ocular transduction in mice by scAAV than the ssAAV counterpart [389, 390]. Successful transduction using scAAV was also described in hematopoietic stem cells [391], brain [392] and liver [393]. However, there is one significant limitation associated with scAAV, \textit{i.e.}, its packaging capacity is half the capacity of the ssAAV. This precludes scAAV to be used in the gene therapy of diseases associated with large size gene abnormalities [394].

1.2.6 Production of rAAV
To overcome the problem of Ad contamination, the standard protocol to generate rAAV involves co-transfection of cells with a vector DNA, \textit{i.e.}, a transgene flanked by the AAV ITRs, a packaging plasmid encoding the \textit{rep} and \textit{cap} genes (without ITRs) and a helper plasmid that provides Ad helper genes [395, 396]. Progress has been made to improve the helper plasmid and pXX6, which contains a mini Ad-genome capable of propagating AAV in the presence of the AAV Rep and Cap protein was developed [397]. pXX6 no longer codes for the hexon, penton, core protein, DNA polymerase, Ad terminal protein and the Ad major late promoter while it contained E21, E4 and VA RNA genes [397]. Combining the AVV vector cassette and the packaging gene cassette into a single plasmid so that both genes can be equally taken by the cells during transfection has also been tried. However, there was no significant difference in the rAAV titre when the two cassettes were used in a single plasmid [397]. Although triple transfection of HEK 293 cells is the standard protocol for AAV production different approaches including the use of i) a baculovirus expression system [398-400], ii) a packaging cell line (HeLa derived cell lines), iii) recombinant Ad encoding the complete rAAV genes in its E1 region [401], iv) a HSV amplicon system [402], v) rAAV packaging cell lines containing inducible Ad helper genes [403] and vi) a two plasmid- (AAV vector and a plasmid that contains AAV Rep and Cap and Ad helper genes) based production [404] have been reported.
1.2.7 Purification of rAAV
Recovery of rAAV from the packaging cell lines involves liberating rAAV from the cells by freeze-thaw cycles, separating rAAV from other cell and media components and concentrating the product. Cellular components can be removed by either centrifugation or filtration [405].
Cesium chloride (CsCl) gradient centrifugation is the commonly employed method of purifying rAAV [406]. Although CsCl purification yields highly pure virus preparations, this yields small scale viral lots suitable for research applications, but the limited availability of ultracentrifuges and a requirement for extensive dialysis of viral preparations due to CsCl toxicity. This results in variable quality of vector preparations, significant loss of infectivity and aggregation in storage [407].
Iodixanol gradient centrifugation is an alternative to CsCl. As it has a much lower toxicity compared to CsCl iodixanol prevents aggregation of rAAV particles and does not reduce infectivity [400, 407].
Chromatographic methods were also developed and used in different studies. These methods increased vector purity, biological potency and throughput [408, 409]. Potter et al. developed a simple chromatographic method to purify rAAV 2, 8 and 9 [410]. They utilized flocculation of cell debris under low pH condition for partial purification followed by single step cation exchange chromatography via a sulfopropyl column. The flocculation step eliminated the bulk of the contaminating protein and DNA allowing AAV binding to and subsequent elution-from the resin [410]. One step affinity chromatography was also used to purify rAAV produced from insect Sf9 cell lines that contain rep and cap genes that are induced by baculovirus [398, 399].

1.3 Tissue Resident Memory T (TRM) cells

1.3.1 Introduction to memory T cells
Immunological memory follows the encounter and control of infectious disease by the immune system. When pathogen-specific immune cells are activated they express certain migration
receptors allowing them to home to the site of infection where they contribute to the control of infection [411]. Following successful control of infection, the majority of pathogen specific immune cells will die, but a few of these antigen experienced cells will survive in the host and become memory cells [412].

CD8+ T cells are principal components of this process and protect against pathogens that invade intracellular compartments [413]. Memory CD8+ T cells are present within secondary lymphoid organs (SLO), blood, and the nonlymphoid tissues (NLT), as well as primary lymphoid organs such as thymus and bone marrow [413].

Based on their surveillance pattern, memory CD8+ T cells isolated from human blood are divided into two; central memory T (T_CM) cells and effector memory T (T_EM) cells [414]. T_CM cells express lymph node homing receptors, CD62L and CCR7, but have limited immediate effector function while T_EM cells lack lymph node homing receptors but express non-lymphoid tissue target receptors which enable them reach peripheral organs, where they can act against foreign agents [415, 416]. Functionally T_CM cells are important for long term protection being involved in secondary responses while T_EM cells are immediate responders [417].

Memory T cells can persist for a lifetime in the absence of antigen and even MHC molecules. One example of memory lymphocyte persistence was shown among individuals vaccinated with vaccinia virus vaccine. In these group of volunteers’ cellular immunity against vaccinia virus was maintained for decades after the vaccination, with an estimated half-life of 8-15 years [418]. On the other hand, a study in MHC-I deficient mice revealed that CD8+ memory T cells can persist in these mice once they differentiate in to memory T cell subset [419]. Later it was shown that T cell receptor (TCR) expression was not mandatory for the maintenance of memory CD8+ T cells [420].
The mechanism of memory maintenance and lineage relationship between memory T cells is not clearly known. Two models were proposed regarding the development of memory T cells. The first is the linear differentiation model, which predicts that memory cells are the progeny of effector cells that escaped activation induced-cell death [421, 422]. A study by Wherry et al. reported $T_{CM}$ cells and $T_{EM}$ cells as a non-distinctive group of cells [423]. According to this report, following pathogen clearance in mice infected with lymphocytic choriomeningitis virus or *Listeria monocytogenes*, $T_{EM}$ cells were converted to $T_{CM}$ cells. In a more continuous manner, following acute infection, $CD8^+$ T cells differentiation follows this path; naïve cells will differentiate into effector cells which then give rise to $T_{EM}$ cells. $T_{EM}$ cells will then differentiate into $T_{CM}$ cells [423].

The second model of memory T cell development suggested that memory T cells directly arise from naïve cells (non-linear differentiation) [421]. In this model $T_{EM}$ cells and $T_{CM}$ cells were described as a completely distinct group of cells [424]. A TCR repertoire study in an adoptive transfer rat model indicated that 75% of $T_{CM}$ cells and $T_{EM}$ cells were derived from a common naïve progenitor cell [424]. Complementing theory, Manjunath and colleagues reported that effector differentiation is not a prerequisite for memory cell differentiation suggesting that the development of memory T cells occurs directly from antigen-activated $CD8^+$ T cells without passing the effector stage [425].

Unlike naïve T cells, memory cells migrate between the blood, the lymph and tissues [414, 426]. The migration pattern of memory T cells and the molecules involved in the process of migration contributed to the heterogeneous phenotype of memory T cells [427]. The migration and distribution of an antigen-specific memory T cell population depends on factors like the site of infection and the type of tissue that is colonized by the antigen [428]. It was shown that pathogen-specific T cells persist in the lungs of mice for over a year after respiratory virus infection; these
cells were present in high number and were distinct from the cell population in the spleen and peripheral lymph nodes [429]. Similarly, HSV type 2 (HSV-2) specific CD8+ T cells express a surface molecule, cutaneous lymphocyte associated antigen (CLA), that enable trafficking to the skin [430]. In contrast, CD8+ T cells specific to non-skin tropic herpesvirus lack CLA [430]. These studies and other similar reports [431, 432] showed the persistent presence of transcriptionally and phenotypically unique memory T cells in specific tissues and these types of memory cells were described as tissue resident memory (T_{RM}) cells which form a third group of memory T cells [433].

1.3.2 Tissue Resident Memory T (T_{RM}) cells
T_{RM} cells are subsets of memory T cells that are maintained in tissues and organs independently of circulating memory T cells (T_{CM} and T_{EM} cells) [434]. T_{RM} cells are important in protection against infection in sites where circulating memory T cells are less effective [435]. T_{RM} cells express the integrin subunit CD103, which is not found on their circulating counterparts [436]. However, this is not universal to all T_{RM} cells in all tissues and organs as CD103−T_{RM} cells are found in the intestinal lamina propria [437, 438]. In addition, CD103 was not upregulated in T_{RM} cells isolated from SLO as compared to T_{RM} cells isolated from the small intestine [439]. T_{RM} cells are phenotypically distinct from other memory T cells; they highly express CD69, an activation marker transiently upregulated in activated T cells, and CD49 (Table 3.1) [433, 434, 440]. Functionally T_{RM} cells are highly cytotoxic and antiviral than other memory T cell subsets which is due to high affinity TCR [441].

The proof of existence of T_{RM} cells is mainly based on parabiosis, a procedure of joining two animals so that they share each others blood circulation [442], and transplant experiments used to study memory CD8+ T-cell trafficking [443]. To show that T_{RM} cells are non-migratory, Gebhardt et al. first transplanted dorsal root ganglia, latently infected with HSV, to under the kidney capsule of a recipient mouse that had circulating HSV-specific CD8+ T cells and this
resulted in the migration of HSV-specific CD8$^+$ T cells from the circulation to the graft [444]. Next the grafted ganglia were extracted and transplanted to a naïve, secondary host. Simultaneously fresh latent ganglia were transplanted into the secondary host at a different site of the kidney capsule. Nine days after the transplantation, grafts were removed and infiltrating cells were analysed; it was found that only recruited CD8$^+$ T cells were found in the fresh graft in contrast to the T$_{RM}$ cells carried by the original graft suggesting that these cells do not egress to the circulation [444]. The presence of T$_{RM}$ cells in the skin was also shown by parabiosis and transplantation studies in mice. These cells remained in the skin and were unable to recirculate [445]. Adoptive transfer of CD8$^+$ T cells from male to female showed that CD8$^+$ T cells with the ability to migrate into the skin survived rejection by the recipient immune system. These cells were those which persisted in the skin following infection, suggesting that these cells escape immune clearance because they were able to enter to the skin rather than entering the circulation [446].

T$_{RM}$ cells exhibit limited motility [446] and they appear non-functional when experimentally returned to the circulation, in contrast to their CD103$^-$ counterparts [447]. Non-recirculating CD103$^+$ T$_{RM}$ cells have now been described in a variety of tissues and organs, including the brain, intestine, skin, and sensory ganglia [444, 447, 448], and CD103 CD8$^+$ T cells in the salivary glands [449] and potentially the lungs [450-452] are likely to belong to this class of resident memory cells.

1.3.2.1 Maintenance of T$_{RM}$ cells
Maintenance of T$_{RM}$ cells within localized tissues depend on their ability to adapt local survival strategies, resist shedding into the lumen at mucosal epithelial surfaces and ignore egress signals [453, 454]. Although the requirements for retention of T$_{RM}$ cells is not universal some of the proposed mechanisms and signals are discussed in the following paragraphs.
Table 1.2. Memory T cell subsets [453]

<table>
<thead>
<tr>
<th>Subset</th>
<th>Phenotype</th>
<th>Location</th>
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<tr>
<td>T&lt;sub&gt;CM&lt;/sub&gt; cells</td>
<td>CD&lt;sub&gt;44&lt;/sub&gt;&lt;sup&gt;high&lt;/sup&gt;, CD&lt;sub&gt;62L&lt;/sub&gt;&lt;sup&gt;+&lt;/sup&gt;, CCR&lt;sub&gt;7&lt;/sub&gt;&lt;sup&gt;+&lt;/sup&gt;, CD&lt;sub&gt;127&lt;/sub&gt;&lt;sup&gt;+&lt;/sup&gt;, CD&lt;sub&gt;69&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;, CD&lt;sub&gt;103&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Lymph nodes, spleen (white pulp &gt; red pulp), blood, and bone marrow</td>
</tr>
<tr>
<td>T&lt;sub&gt;EM&lt;/sub&gt; cells</td>
<td>CD&lt;sub&gt;44&lt;/sub&gt;&lt;sup&gt;high&lt;/sup&gt;, CD&lt;sub&gt;62L&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;, CCR&lt;sub&gt;7&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;, CD&lt;sub&gt;127&lt;/sub&gt;&lt;sup&gt;+&lt;/sup&gt;, CD&lt;sub&gt;69&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;, CD&lt;sub&gt;103&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Spleen (red pulp &gt; white pulp); fewer in lymph nodes, blood, lung, liver, intestinal tract, reproductive tract, kidney, adipose tissue, and heart</td>
</tr>
<tr>
<td>T&lt;sub&gt;RM&lt;/sub&gt; cells</td>
<td>CD&lt;sub&gt;44&lt;/sub&gt;&lt;sup&gt;high&lt;/sup&gt;, CD&lt;sub&gt;62L&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;, CCR&lt;sub&gt;7&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;, CD&lt;sub&gt;11a&lt;/sub&gt;&lt;sup&gt;high&lt;/sup&gt;, CD&lt;sub&gt;69&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;, CD&lt;sub&gt;103&lt;/sub&gt;&lt;sup&gt;+&lt;/sup&gt;</td>
<td>epithelium of the skin, gut, and vagina; salivary glands and lung airways; brain and ganglia (T&lt;sub&gt;RM&lt;/sub&gt; cells isolated from lamina propria, SLO and liver showed a distinct pattern of CD44 and CD103 [439, 455])</td>
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The integrin α<sub>e</sub>β<sub>7</sub>, also known as CD103, one marker associated with T<sub>RM</sub> cells, can bind to E-cadherin, a protein that tethers epithelial cells together via a homophilic interaction that forms tight junctions. It is hypothesized that α<sub>e</sub>β<sub>7</sub> allows CD8<sup>+</sup> T cells to adhere to the E-cadherin expressed on epithelial cells which supports long term survival [447, 456, 457]. On the other hand, α<sub>e</sub>β<sub>7</sub> CD8<sup>+</sup> T cells in the brain expressed more B-cell lymphoma-2 protein family (Bcl-2), a family of prosurvival proteins that inhibit cell death [458], early after infection making α<sub>e</sub>β<sub>7</sub> expression a key protein in CD8<sup>+</sup> T cell survival [435, 447]. In contrast, others reported that α<sub>e</sub>β<sub>7</sub> is necessary for CD8<sup>+</sup> T cell accumulation in the small intestine epithelium following oral infection with <i>L. monocytogenes</i> infection, but long-term survival was not dependent on this molecule [459]. Other reports state T<sub>RM</sub> cells in different organs do not express α<sub>e</sub>β<sub>7</sub> [439, 460].
From these observations it can be concluded that while αeβ7 expression on antigen specific CD8+ T cells might be strongly indicative of residence, but it is likely that many T_{RM} cells do not depend on, nor even express, αeβ7 [461].

Sphingosine-1 phosphate receptor 1(S1PR1) mediates T cell egress in lymph nodes by inducing chemotaxis to sphingosine-1- phosphate (S1P) present within efferent lymph [462]. Thus, downregulation of S1P responsiveness might be one of the mechanisms for the maintenance of TRM cells [461]. S1PR1 downregulation is associated with cell surface expression of the C-type lectin CD69. It has also been shown that CD69 deficient (CD69−/−) mice had reduced number of T_{RM} cells in the skin epidermis or lungs [450, 463]. In another study it was reported that transcription factor KLF2, that promotes expression of the genes encoding S1PR1, was downregulated in CD8+ T cells in NLTs while forced expression of S1PR1 prevented the establishment of T_{RM} cells [464]. To conclude, the downregulation of S1PR1 and KLF2 and upregulation of CD69 might play important roles in the development and maintenance of T_{RM} cells [461].

Other mechanisms for the retention of T_{RM} cells have also been described. T-box transcription factor (TF) is known to influence the development of innate and adaptive immune system [465]. To assess the impact of T-box TF, Mackay et al. used a HSV skin infection model. This study showed that as virus-specific CD8+ T cells progress to the stage of becoming CD103+, T-box TF expression was downregulated and forced expression of T-box TF prevented formation T_{RM} cells in the skin [466]. Observations from this study also showed that there was a reciprocal downregulation of T-box and transforming growth factor (TGF)-β, which has been shown to promote the development of T_{RM} cells [456, 466]. In conclusion, the downregulation of T-box TFs is important for TGF-β mediated cytokine signaling that supports the development and maintenance of T_{RM} cells.
Other factors including CD4+ T cell help, regulatory T cells (Tregs), transcription factor aryl hydrocarbon receptor have also been investigated for their role in the development and maintenance of T\textsubscript{RM} cells [467-469].

1.3.2.2 Role of T\textsubscript{RM} cells in bacterial and parasitic infection

Successful elimination of intracellular bacterial and parasitic pathogens requires the generation of a specific T cell response. T\textsubscript{RM} cells have been reported to be involved in the elimination of these pathogens [459, 470].

It has been shown that an attenuated *Plasmodium falciparum* sporozoite vaccine was protective against controlled human malaria infection at least for 1 year, and studies to assess the immune correlates in non-human primates showed the presence of long-lived INF-γ producing CD8+ T cells in the liver rather than in the blood, and these cells were regarded as T\textsubscript{RM} cells [471]. An attempt to expand malaria-specific T\textsubscript{RM} cells in the liver of mice showed a distinct population of CD8+ T cells expressing CD69+ (T\textsubscript{RM} cells) following vaccination of mice using *Plasmodium berghei* radiation attenuated-sporozoites [455]. These liver T\textsubscript{RM} cells induced a sterilising immunity against liver stage *P.berghei* sporozoite challenge and protected against malaria infection [455].

Intratracheal vaccination with *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) vaccine resulted in infiltration of airways by T cells that expressed tissue residency markers like CD103 and CD49a. Adoptive transfer of air-way resident T cells into naïve mice confers protection against *Mycobacterium tuberculosis* challenge [472].

The role of T\textsubscript{RM} cells was also investigated in infections caused by *L. monocytogenes*. CD103-expressing CD8+T cells accumulated in the intestinal mucosa following oral infection of mice by *L. monocytogenes* [459]. To investigate the role of these cells in protection, mice were first treated with anti-α\textsubscript{4}β\textsubscript{7}, to prevent migration of cells into intestinal mucosa [473]. Treatment with anti-
resulted in a reduced population of T\textsubscript{RM} cells in the intestinal mucosa while circulating CD8\textsuperscript{+} T cell population remain unaffected. Subsequent oral \textit{L. monocytogenes} challenge of mice resulted in a high bacterial burden [459].

1.3.2.3 Role of T\textsubscript{RM} cells in viral infections

The role of T\textsubscript{RM} cells in viral infections was reported in the context of different viruses including influenza virus, HSV and HPV, and their protective capabilities was demonstrated in the skin, genitourinary tract and the lungs [429, 433, 447, 474-476]. Virus specific T\textsubscript{RM} cells were shown to possess high affinity TCR making them highly sensitive to antigens which is required for frontline defence [441].

In a HSV skin infection model, following acute cutaneous infection with HSV, virus-specific CD8\textsuperscript{+} T\textsubscript{RM} cells were induced [444]. These cells were able to control secondary infection with the virus only in the skin area that was previously infected with virus indicating these cells were non-migratory cells [444]. In an attempt to induce T\textsubscript{RM} cells in the skin, Mackay \textit{et al.} transferred \textit{in vitro} activated gBT-I T cells into C57BL/6 mice and treated one flank of the mice with the contact-sensitizing agent 2,4-dinitrofluorobenzene (DNFB) to causes non-specific inflammation that can potentially recruit virus specific T cells in to the skin [435]. The results showed a greater number of T\textsubscript{RM} cells in the DNFB treated flank. Subsequent challenge with HSV showed protection only in the DNFB treated flank, that contained T\textsubscript{RM} cells, while mice with only circulating CD8\textsuperscript{+} T cells developed skin lesions and had higher viral titre [435].

In another study, vaccinia virus skin infection has been shown to generate CD8\textsuperscript{+} T\textsubscript{RM} cells in the skin [445]. To assess the role of these T\textsubscript{RM} cells in infection control, a previously infected mouse was parabiotically joined with an uninfected mouse. After separation each mouse was challenged with vaccinia virus. Remarkably, despite the high frequency of T\textsubscript{CM} cells in the uninfected parabiont these cells were unable to clear the infection until 26 days post infection while the infected parabiont that had T\textsubscript{RM} cells in their skin completely cleared the virus in 6 days [445].
1.3.2.4 Targeting TRM cells in vaccination

In the next few paragraphs the importance of targeting TRM cells in vaccine development is described in the context of HCV and HIV. Viral infections such as HIV and HCV account for a significant level of morbidity and mortality across the world. As these two viral infections share common transmission routes the comorbidities are also significant and the worldwide prevalence of HIV-HCV co-infection is 2, 278, 400 people [477].

Anti-retroviral therapy (ART) and DAAs have contributed significantly to prolonging the lifespan and curing of HIV- and HCV-infected individuals respectively [478] but the annual incidence of HIV and HCV infections is still rising by millions. Furthermore, only 17 million (< 50%) people have access to ART [359] and only 20% of patients are diagnosed for HCV [7] which make meeting the UN 90-90-90 targets for HIV and WHO Sustainable Development Goals for eliminating HCV extremely challenging. Issues involving drug resistance, reactogenicity associated with life-long ART and the lack of universal access to testing and cost-subsidised therapies minimise the ability of effective anti-viral drugs to end the HIV and HCV epidemics [288]. Thus, there is an urgent need to develop effective vaccines to complement the eradication efforts of ART and DAA.

Vaccines are of great public health importance and the previous success of the smallpox and poliovirus vaccines and the most recent news release on the successful elimination of measles from Singapore and rubella from Australia, Brunei Darussalam and Macao SAR (China) (http://www.who.int/westernpacific/news/detail/31-10-2018-singapore-wipes-out-measles-australia-brunei-darussalam-and-macao-sar-%28china%29-%28china%29-eliminate-rubella) suggest that vaccination is a cost-effective means to eliminate HIV and HCV infections worldwide [243].

Unfortunately, designing a vaccine for these viruses has been particularly challenging because of the mutable nature of the pathogen and complexity of the immune system involved during these
infections [443]. It is known that T cell responses are vital for the control of these viruses in non-human primates and humans [136, 147, 160, 479-484].

The role of circulating memory T cells in protection from systemic infection that result accumulation of pathogens in lymphoid tissues is immense. However, the ability of these cells to contain localised infections in specific organs or tissues is limited [428]. One of the main reasons for this is the delay in T cell recruitment from the circulation providing an advantage to the pathogen to establish infection before the action of the immune system [411]. To overcome this problem vaccines that can induce localised immunity represent a potential strategy. To this T<sub>RM</sub> cells play the major role in providing protection in localised tissues [485-489]. Vaccines that elicit T<sub>RM</sub> cells in the liver and the mucosal areas of the genitourinary tract could potentially be the future of vaccine development against HCV and HIV respectively.

From some of the studies [413, 455] discussed in (section 1.3.2.2 and 1.3.2.3) and elsewhere [490] it is clear that T<sub>RM</sub> cells play important roles in the immune response against hepatotropic pathogens suggesting that T<sub>RM</sub> cells in the liver against HCV can be potential immune cells to prevent persistent HCV infection.

HIV transmission occurs mainly via the genitourinary tissues and intestinal epithelial region. Hence, localised immunity in these regions is necessary to prevent viral entry and transmission. There are also reports showing the induction of antigen specific T<sub>RM</sub> cells following infection or vaccination with HIV vaccines [491-493]. In human rectosigmoid tissue, abundant CD8<sup>+</sup> T<sub>RM</sub> cells with a robust response against HIV-1 were reported in individuals who controlled HIV-1 infection [493]. On the other hand, a recombinant influenza virus expressing HIV-1 gag protein p24 was able to induce both systemic and mucosal HIV specific CD8<sup>+</sup> T cell response in mice following intranasal vaccination [494]. An intravaginal boost with this vaccine resulted in robust and sustained immune response in the vagina [494]. A further study on the intranasal priming
followed by intravaginal boost indicated that immune cells induced by this strategy were CD103+ CD8+ T cells, which are presumably T<sub>RM</sub> cells [491]. These cells were largely found in the vaginal epithelia and their activation by a cognate antigen in the vagina resulted in the recruitment of both innate and adaptive immune cells [491]. To complement the above studies, Zaric et al. reported the establishment of long lived antigen-specific T<sub>RM</sub> cells in the mucosal tissues of female genitalia and respiratory tract following micro-needle array-mediated vaccination of mice with recombinant Ad5 encoding HIV-1 gag [492]. An intravaginal boost, 150 days after Ad5-gag, with CpG adjuvanted immunodominant HIV-1 CN54 gag<sub>308–318</sub> epitope increased the frequency of these cells [492]. These reports suggest the possibility of inducing local immunity that can act as a frontline defence against the virus.

**1.3.2.5 Prime/Trap vaccination strategy to induce T<sub>RM</sub> cells**

It has been shown that a robust population of antigen-specific CD8<sup>+</sup> T cells can be established in the genital tract following a vaccination strategy termed prime-pull [495]. In this strategy transgenic mice were subcutaneously (SC) immunized with attenuated HSV-2 to activate HSV-2 specific CD8<sup>+</sup> T cells. Five days later chemokines CXCL9 and CXCL10 were applied topically to the vaginal cavity [495] to mediate recruitment of effector CD8<sup>+</sup> T cells to infected tissues via the chemokine receptor CXCR3 [496]. According to this study, SC immunization (prime) with attenuated HSV-2 generates a robust systemic virus-specific CD8<sup>+</sup> T-cell response that was comparable to intravaginal immunization. When the CXCL9 and CXCL10 were applied topically to the vagina (pull) robust recruitment of virus-specific CD8<sup>+</sup> T cells was observed, and these cells were able to establish a resident population [495]. One important observation from this study was that T<sub>RM</sub> cells induced by the prime and pull strategy were protective against HSV challenge. This prime and pull strategy or prime-boost strategy [491] or prime-trap strategy [455] used in different studies revealed that the strategy was able to expand T<sub>RM</sub> cells.
Collectively the above observations indicate that a strategy to prime antigen specific CD8+ T cells and attracting these cells to a specific area of tissue or organ (liver) might be an effective way of generating an effective HCV vaccine.
1.4 Experimental Aims

The main aim of the work described in this thesis is to develop a prime-trap strategy that can result in the appearance of \( T_{RM} \) cells in the liver, that are likely to be highly protective, following ID vaccination of mice with a DNA vaccine encoding NS5B of HCV gt3a and the cytolytic protein, PRF, (pVAX-NS5B-SV40-PRF) to prime T cells followed by IV vaccination with rAAV encoding the vaccine antigen (NS5B) and eGFP (rAAV-NS5B-2A-eGFP) to trap primed T cells.

The specific aims of the project are

1. To prepare and purify rAAV-NS5B-2A-eGFP
2. To identify immunodominant peptides of NS5B of gt3a HCV that can be recognized by T cells in the liver and spleen for tetramer synthesis
3. To assess CMI following prime/trap vaccination strategy
4. To characterise CD8\(^+\) T cells elicited by the prime/trap vaccination strategy
2 Chapter 2: Materials and Methods

A list of reagents, list of equipment and list of kits is provided in Appendix I, II and III respectively. The formulae for bacterial and mammalian cell growth media are provided in Appendix IV and V. A list of labelling dyes and antibodies, buffers and primers is provided in Appendix VI, VII, and VIII respectively.

2.1 Bacterial strains, supplements and storage

2.1.1 Bacterial strains

2.1.1.1 Escherichia coli DH5α cells

Genotype: F− Φ80lacZΔM15 Δ (lacZYA-argF) U169 recA1 endA1 hsdR17 (rK−, mK+) phoA supE44 λ− thi-1 gyrA96 relA1. This strain of E. coli was used for the propagation of DNA vaccines (pVAX-NS5B-PRF and pVAX-PRF) and p5E1VD2/8 plasmid.

2.1.1.2 SURE-2 super-competent cells

Genotype: e14-(McrA−) Δ (mcrCB-hsdSMR-mrr) 171 endA1 gyrA96 thi-1 supE44 relA1 lac recB recJ sbcC umuC::Tn5 (Kanr) uvrC [F’ proAB lacIqZΔM15 Tn10 (Tetr) Amy Camr}(Catalogue # 200152, Stratagene, Agilent technologies)

This strain, “Stop Unwanted Rearrangement Events” (SURE), allows the cloning of certain DNA segments that are “unclonable” in conventional E. coli strains. The SURE strain lacks components of the pathways that catalyse the rearrangement and deletion of non-standard secondary and tertiary structures, including cruciforms (caused by inverted repeats) and Z-DNA, that occur frequently in eukaryotic DNA and that impede the cloning of the eukaryotic DNA in conventional strains (https://www.agilent.com/cs/library/usermanuals/public/200256.pdf). Cloning of genes into pAM2AA vector and propagation of plasmid pXX6 was performed in these cells.

2.1.2 Preparation of heat competent E. coli

Competent DH5α E. coli or SURE 2 super-competent cells were produced for bacterial transformations. The bacterial cells were streaked out from a glycerol stock onto a Psi-b agar
plate containing no antibiotics, and incubated for 20 hours at 37°C. The following day a single colony was picked and grown in 10 ml of Psi-a media at 37 °C for 4 hours (until OD$_{550}$ = 0.3). This starter culture was diluted 1:20 in 100 ml Psi-a medium and incubated at 37°C for a further 3 hours (until OD$_{550}$ = 0.48). The bacterial culture was then chilled on ice for 5 minutes and pelleted by centrifugation at 4500 revolution per minute (rpm) for 5 minutes at 4 °C. The supernatant was removed and the bacterial cells were resuspended in 40 ml chilled TfbI solution. The cells were incubated for a further 5 minutes on ice and then centrifuged at 4500 rpm for 5 minutes at 4 °C. The supernatant was removed, the cells were resuspended in 4 ml of chilled TfbII solution and incubated for 15 minutes on ice. The cells were then aliquoted into 50 μl volumes and snap frozen in liquid nitrogen. The aliquots were stored at -80 °C until required.

2.1.3 Supplement
Ampicillin (stock solution of 100 mg/ml) or Kanamycin (stock solution 50 mg/ml) was added to Luria-Broth (LB) media at 1:1000 dilution when necessary. The final concentration of the antibiotics was 0.1 mg/ml and 0.05 mg/ml, respectively.

2.1.4 Glycerol stocks of bacteria
For long term storage of transformed bacteria, glycerol stocks were produced by adding 0.5 ml of an overnight bacterial culture in antibiotic-supplemented LB media to 0.5 ml of sterile 40% glycerol in an eppendorf tube. The glycerol stocks were stored at -80°C.

2.2 In vitro techniques

2.2.1 pAM2AA-eGFP plasmid
pAM2AA-eGFP was a gift from Dr. David Bowen (Centenary Institute, Sydney). This plasmid was constructed by inserting eGFP into pAM2AA plasmid which in turn was constructed by removing the cap and rep genes from the AAV genome leaving only the cis acting inverted terminal repeats (ITR). Flanked by the ITRs, the vector contains a liver specific enhancer and promoter region, viz. two ApoE enhancers and the human α-1 anti-trypsin promoter (hAAT), a
multiple cloning site (MCS) for restriction enzyme digestion and insertion, the enhanced green fluorescent protein (eGFP) reporter gene, a woodchuck hepatitis virus post-transcriptional regulatory element (WPRE), the Bovine Growth Hormone poly adenylation (BGH PolyA) site that terminates transcription and an ampicillin resistance selectable marker (Figure 2.1). The codon optimised sequence of HCV gt3a NS5B was cloned into the MCS under the control of the AopE/hAAT enhancer and promoter, upstream of the eGFP gene. Protease cleavage between NS5B and eGFP was achieved by introducing the foot and mouse disease virus 2A (FMDV2A) protease.

Figure 2.1 A schematic map of pAM2AA (A) and pAM2AA-eGFP (B) showing the AAV-ITRs, ApoE enhancer, hAAT promoter, multiple cloning site (MCS), WPRE, and BGH polyadenylation site (pA) (Generated using SnapGene®)

### 2.2.2 PCR primer design and gene amplification

Primers were designed to amplify an entire gene of interest and introduce a start codon (ATG), a stop codon and/or a Kozak sequence (GCCACC) when necessary. Primers also introduced restriction enzyme sites to allow subsequent digestion and ligation of the PCR product into a plasmid. See Appendix IX for all primer sequences.
Primer sequences were sent to Geneworks Pty Ltd or Integrated DNA Technologies (IDT) Inc. for synthesis. PCR amplification of genes of interest was performed on a Peltier PTC-200 Thermal Cycler using the Kapa High Fidelity (HiFi) Taq polymerase enzyme kit (Geneworks # KP-KK2102). Each reaction mixture contained 1X KAPA HiFi buffer, 0.3 mM of each of the dNTPs, 0.3 mM forward primer, 0.3 mM reverse primer, 10-50 ng of template DNA and 0.5 U KAPA HiFi DNA polymerase adjusted to a final volume of 50 µl with MQ H₂O. Each reaction was performed as per the following cycling conditions (Table 2.1).

**Table 2.1. Cycling conditions for PCR**

<table>
<thead>
<tr>
<th>Cycling</th>
<th>Initial cycle</th>
<th>x35 cycles</th>
<th>Final cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (ºC)</td>
<td>Denaturation</td>
<td>Denaturation</td>
<td>Annealing</td>
</tr>
<tr>
<td>98</td>
<td>93</td>
<td>Tm*</td>
<td>72</td>
</tr>
<tr>
<td>Time</td>
<td>2 minutes</td>
<td>20 seconds</td>
<td>15 seconds</td>
</tr>
</tbody>
</table>

*Annealing temperature was Tm for KapaTaq Hifi and Tm-5 for KapaTaq

### 2.2.3 Gel electrophoresis and DNA purification

PCR products were analysed by gel electrophoresis on 0.8-1% w/v agarose gel in Tris-Borate EDTA (TBE) buffer (0.22M Trizma base, 180mM Boric acid, 5mM EDTA). DNA bands were visualized using GelRed (Biotium #41003) and a BioRad GelDoc imager and compared to a molecular weight standard DNA ladder (New England Biolabs NEB 100bp, 1000bp, or 2500bp) to confirm that the DNA amplicon was the correct size. The correct band size that contained the amplified gene was purified using the PureLink® Quick Gel Extraction and PCR purification.
Combo Kit (Life Technologies #K3100-01). Excised gel slices that contained the correct size DNA band were dissolved in solubilisation buffer at a ratio of 1:3 and incubated at 50°C for 15 minutes. The dissolved DNA was then mixed with isopropanol to increase DNA yield. The DNA was then bound to the quick spin column by centrifugation at 13,000 rpm for 1 minute. The column was washed once with 500µl of wash buffer and centrifuged again at 13,000rpm for 1 minute. DNA was eluted from the column by adding 30-50µl of elution buffer and centrifuging at 13,000rpm for 1 minute.

2.2.4 Digestion and Ligation

The PCR product of the gene of interest and the plasmid backbone were digested by an appropriate restriction endonuclease (NEB) at 37°C overnight to ensure complete digestion. The digested products were purified using PureLink® Quick Gel Extraction and PCR purification Combo Kit (Life Technologies #K3100-01) as described in section 2.2.3. The pAM2AA vector was de-phosphorylated using Antarctic phosphatase (NEB #M0289). The phosphatase reaction mix that contained digested, purified vector, 1 U phosphatase and 1X phosphatase buffer was incubated for 20 minutes at 37°C. The phosphatase was heat inactivated at 65 °C for 15 minutes.

To ligate the digested components, the insert and vector were mixed at a ratio of 1:1, 3:1 and 5:1 keeping the final amount of DNA to 100 ng. The mixture was incubated at 16°C overnight in the presence of T4 ligase (NEB #M0202). The ligated mixture was transformed into SURE 2 super competent E. coli, plated on to LB agar containing ampicillin and grown at 37°C overnight. Colonies from positive plates were screened using colony PCR for the presence of the cloned gene and positive colonies were grown overnight in LB containing ampicillin at 37°C on a shaker. Plasmid DNA mini-prep was prepared (Section 2.2.6) and digested using appropriate restriction enzymes and analysed by agarose gel electrophoresis to confirm the presence of the gene of interest.
2.2.5 DNA Sequencing
The gene of interest in the plasmid was sequenced using a forward primer that anneals to the hAAT promoter region and a reverse primer that anneals to the 5’ end of the eGFP gene. Sequencing reactions were performed using Big Dye terminator mix (IMVS) using 1.2ul of 10uM forward or reverse primer and 250ng of DNA. The BLAST nucleotide alignment tool was used to confirm that the sequence in the plasmid DNA matched the sequence of the gene of interest.

2.2.6 Plasmid DNA amplification and purification
Briefly, transformed E. coli cells were grown for 16 hours at 37 °C on a shaker in antibiotic-supplemented LB media. The bacteria were pelleted by centrifugation and resuspended, then the bacteria were lysed with lysis buffer. The lysis buffer was neutralised by neutralisation buffer. All the buffers were provided in the kits, described below.

The miniprep was prepared using PureLink® plasmid miniprep kit (ThermoFisher Scientific). Bacterial debris was removed by centrifugation at 12000 rpm for 10 minutes and the supernatant was added to the silica spin column to bind and purify the DNA. The column was washed with the buffer provided and the DNA was eluted in the elution buffer.

The endotoxin-free DNA mega-preparation was prepared using Qiagen® Mega prep kit (Qiagen #12191). In this case bacterial debris was removed using a vacuum by passing the bacterial lysate through a filter provided in the kit. The flow-through was added to a gravity-flow column to bind DNA. The column was washed, the DNA was eluted in elution buffer and then precipitated using isopropanol. The DNA was pelleted by centrifugation at 4000 rpm for 90 minutes and washed with 70 % ethanol. The DNA pellet was then air-dried and dissolved in endotoxin free TE-buffer. The concentration of DNA was determined using Nanodrop2000 spectrophotometer (ThermoFisher Scientific) and DNA was considered as “pure” when the absorbance (A) ratio at A 260/280 was ~ 1.8.
2.2.7  **Cell culture and transfection**

2.2.7.1  **Cell culture**
Human Embryonic Kidney (HEK 293T) cells and HT1080 cells were cultured in Dulbecco’s modified Eagles medium (DMEM) supplemented with 10% heat inactivated foetal calf serum (FCS) and 1% penicillin/streptomycin and incubated at 37°C in 5% CO₂. Cell cultures were routinely grown in a T75 cell culture flask and passaged at 80-90% confluency.

2.2.7.2  **Cell storage and retrieval**
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. To retrieve cells from liquid nitrogen, vials of cells were thawed in a 37°C water bath and then transferred to a 10 ml falcon tube; 9 ml of pre-warmed media was then added, and the cells were pelleted by centrifugation at 300x g for 5 minutes. The supernatant was removed, the cells were re-suspended in fresh media and plated.

2.2.7.3  **Transient transfection**
Appropriate numbers of cells were seeded on to cell culture plates or flasks and allowed to adhere overnight resulting in approximately 60-70% cell confluency on the day of transfection. Lipofectamine® LTX with Plus™ Reagent from Invitrogen (Thermo Fisher Scientific #15338100) was used to transfect cells in 96, 24 and 6 well cell culture plates. Briefly DNA, diluted with Opti-MEM, was mixed with plus reagent (1µg DNA with 1µl of plus reagent) and incubated for 5 minutes at room temperature. LTX diluted with Opti-MEM was added to the DNA-plus reagent mixture in a 1:1 ratio, incubated for 5 minutes at room temperature and then transferred to the wells. A control well transfected with DNA encoding the reporter gene eGFP was used to confirm transfection efficiency. The transfected cells were incubated for 48-72 hours.
at 37°C in 5% CO₂ and protein expression was assessed by flow cytometry, immunofluorescence staining or direct microscopic examination.

2.2.8 Immunofluorescence staining
Forty-eight hours post-transfection with DNA, cells were fixed with 4% paraformaldehyde for 20 minutes, permeabilised with methanol at -20°C and then blocked with 2.5% Bovine serum albumin (BSA) (Sigma-Aldrich) containing 0.3% Triton X-100 (Sigma-Aldrich) in phosphate buffered saline (PBS) prior to the addition of the primary antibody. HCV gt3a patient pooled serum, representing the primary antibody, was diluted 1 in 300 in 1% BSA, 0.3% Triton X-100 in PBS, added to the cells and incubated for 3 days at 4°C. After washing, the secondary antibody, goat anti-human Alexa 555 (Life Technologies), was added to the cells and incubated for 2 hours at 37°C then washed. The cells were then stained with diamidino phenylindole (DAPI) (Life Technologies) and examined with a Zeiss LSM-700 fluorescent microscope.

2.2.9 rAAV-NS5B-2A-GFP production

2.2.9.1 Preparation of HEK293 T cells
HEK 293 T cells were seeded in T75 tissue culture flasks at a density of 6x10⁶ cells per flask and allowed to adhere overnight resulting in approximately 80-90% cell confluency on the day of transfection.

2.2.9.2 Preparation of polyethyleneimine (PEI)
Ten mg of linear PEI was dissolved in 1ml of PBS (without Ca²⁺/Mg²⁺) by heating at 80°C. Once completely dissolved, it was diluted to 1mg/ml (working solution) with PBS. The working solution was filter sterilized using a 0.2μm filter and used immediately [497, 498]. Fresh PEI was prepared whenever needed for transfection.
2.2.9.3 Triple transfection

rAAV-NS5B-2A-GFP was packaged following triple transfection using PEI as described elsewhere [497]. All plasmid concentrations were adjusted to 1 µg/µl in TE buffer before mixing. The plasmids were mixed at a 3:1:1 ratio of pXX6:pAM2AA-NS5B-2A-eGFP: p5E1VD2/8, i.e., 25.2 µg of pXX6, 8.4 µg of pAM2AA-NS5B-2A-GFP and 8.4 µg of p5E1VD2/8. PEI was added to the DNA mixture at a 3:1 ratio. Accordingly, 126µl of 1mg/ml PEI was added to 42µg total DNA. The transfection mixture was adjusted to a total volume of 1.5 ml DMEM and was vortexed for a few seconds then incubated at room temperature for 15-20 minutes [497, 498]. The mixture was then added to a T75 flask. The flasks were returned to the incubator for 72 hours at 37°C, 5% CO2.

2.2.9.4 Virus harvest and purification

rAAV was harvested and purified using a modified polyethylene glycol- (PEG) 8000/sodium chloride (NaCl) precipitation method described by Guo et al., 2012 [498]. Briefly, the cells were removed by scraping, and the culture media was centrifuged at 3000 × g for 10 minutes to pellet the cells and cell debris. The cells were pooled into one 50 ml conical tube then lysed with 5 ml of lysis buffer (50 mM Tris–Cl; 150 mM NaCl; 2 mM MgCl2, pH 8.0). AAV particles were released from the cells with three freeze/thaw cycles between dry ice-ethanol and a 37°C water bath. Fifty U/ml benzonase and 10 U/ml RNase were added to the solution and incubated for 1hour at 37°C. To remove the cell debris, the cells were centrifuged at 2500 × g for 10 minutes and the supernatant was harvested.

Forty percent PE-8000 (Sigma-Aldrich), 2.5 N NaCl, was added to the supernatant to a final concentration of 8%, 0.5 N respectively. The solution was then incubated overnight at 4°C on a mixer and centrifuged at 12,000 rpm for 1hour. The supernatant was discarded and the pellet containing the virus was resuspended in a minimal volume of 50mM HEPES buffer (pH=8).
The crude rAAV solution was treated with an equal volume of chloroform and vigorously vortexed for 2 minutes at room temperature. The solution was centrifuged at 370 x g using a desktop centrifuge for 5 minutes, and the supernatant was allowed to stand at room temperature for 30 minutes to evaporate the chloroform. The virus-containing solution was filtered through a 0.2µm filter, transferred to an Amicon ultra centrifugal filter (100K MWCO, Amicon) device and concentrated by centrifuging at 3000 rpm for 10 minutes. The concentrated virus was dispensed into 100µl aliquots and stored at -80°C until further use.

**2.2.9.5 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)**

The purity of rAAV-NS5B-GFP was evaluated by electrophoresis in 10% SDS-PAGE as described elsewhere [499, 500]. Five µl of the virus stock was mixed with an equal volume of 2x loading buffer and incubated at 95°C for 5 minutes. The sample was loaded to the gel and electrophoresed at 200 V. The gel was immersed and incubated twice in a pre-staining solution (50% methanol and 10% acetic acid) and stained with 0.25% (w/v) Coomassie Brilliant Blue R250 dissolved in a solution containing 50% methanol (v/v) and 10% glacial acid (v/v), and destained (40% methanol and 8% acetic acid) until clear bands with low background were shown.

**2.2.9.6 Titration of rAAV-NS5B-2A-GFP using quantitative real time-PCR**

The yield of virus following purification was determined by quantitative real time PCR using the AAVpro® titration kit for real time PCR version 2 (TaKaRa, Catalogue # 6233). In this method, PCR amplification is monitored in real time using a fluorescent DNA intercalator. The reagent in this kit, SYBR® Premix Ex Taq ™ II, includes a hot start PCR enzyme and SYBR Green I for fluorescent detection. The quantification was based on the virus ITRs. Briefly 2µl of the purified virus was treated with DNase at 37°C for 20 minutes in a 20 µl final volume. The DNase was inactivated by incubating at 95°C for 10 minutes. Then, 20 µl of AAV lysis buffer (provided with the kit) was added to the sample and incubated at 70°C for 15 minutes.
to release the viral genome. The released viral genome was diluted 50-fold using EASY dilution buffer (provided with the kit) and 5 µl of the diluted sample was used as a template for real time PCR.

The reaction mixture was prepared as follows in a final volume of 25µl; 5 µl of template, 12.5 µl of SYBR® Premix Ex Taq II (2X conc.), 0.5 µl 50X Primer mix (provided with the kit) and 7 µl of dH₂O. The PCR was run in a 3-step cycling condition with one cycle of initial denaturation at 95°C for 2 minutes followed by annealing for 5 seconds at 95°C (35 cycles) and elongation for 30 seconds at 60°C. A positive control (a plasmid DNA that contained the AAV ITR, provided with the kit) at different concentration ranges (2x10² to 2x10⁷ copies/µl) was run with the samples to generate a standard curve.

2.2.9.7 In vitro transduction of HT1080 Cells with rAAV-NS5B-2A-GFP

HT1080 cells were seeded on to a 96 well cell culture plate at a density of 2.5x10⁴ cells per well in 100 µl of DMEM (10% FCS and 1% P/S). On the next day the virus was thawed from -80°C, mixed with DMEM (2% FCS and 1% P/S) and the virus-containing medium was used to replace the growth medium. A final concentration of 5 µM Hoechst 33342 (Invitrogen) was used as a transduction inducing agent [501, 502].

2.2.9.8 RT-PCR for the detection of packaged viral genome

2.2.9.8.1 RNA extraction

HT1080 cells were transduced with rAAV-NS5B-2A-GFP as described above and RNA was extracted using QIAGEN RNeasy mini kit as per the manufacturer’s instructions. Briefly, cells were homogenized with a lysis buffer followed by ethanol (70%) precipitation. The sample was passed through a RNeasy spin column after which it was washed and eluted using RNase free water. The concentration of the RNA was measured using a Nano-drop spectrophotometer and used for cDNA synthesis.
2.2.9.8.2 cDNA synthesis
RNA was converted to cDNA using the SuperScript™ IV VILO™ Master Mix (Invitrogen) following the manufacturer’s instructions. Briefly, 500 ng of RNA template was digested with Genomic DNA wipeout at 42°C for 2 minutes in a final volume of 10 µl. Next, 4µl of SuperScript™ IV VILO™ Master Mix were added and the final volume was adjusted to 20µl using nuclease free water. The master mix contained reverse transcriptase (RT), a proprietary recombinant RNase inhibitor, helper proteins, stabilizer proteins, oligo (dT)18, random hexamer primers, MgCl2 and dNTPs. The mixture was transferred to a thermal cycler and reverse transcription was performed under the following conditions; annealing at 25°C for 10 minutes, reverse transcription at 50°C for 10 minutes and enzyme inactivation at 85°C for 5 minutes. Controls of no RNA and no RT were included.

2.2.9.8.3 PCR
Using the cDNA as a template, the PCR was performed to detect NS5B and eGFP sequences. Multiple primers that can amplify a sequence of not more than 400bp long were designed. These primers encompass the region of NS5B and eGFP in the rAAV genome sequence. A housekeeping gene GAPDH was included as a positive control for transcription. The reaction mix (total volume of 20 µl with RNase-free MilliQ water, 2.5 mM MgCl2, 1X QuantiFast SYBR Green PCR Master mix, 1 µl cDNA, 1 µM of the forward primer and 1 µM reverse primers) was subjected to a 3-step cycling program with initial denaturation at 95°C for 2 minutes, followed by annealing at 95 °C for 5 seconds and elongation (fluorescent detection) at 60 °C for 30 seconds.

2.2.9.9 Fluorescent Target Array (FTA)
2.2.9.9.1 FTA cell preparation
The use of FTA, a technology where autologous spleenocytes from naïve mice are fluorescently bar coded and transferred to vaccinated mice, to assess T cell functionality in vivo was previously described elsewhere [503, 504]. Briefly, splenocytes from age and sex matched naïve mice were
collected and pooled. The cells were counted and evenly distributed into 2 ml aliquots in R-5 (RPMI-1640 (Invitrogen), 5% FCS, 25 mM HEPES, 1 mM Sodium Pyruvate, 1x 2mE, 1% Pen Strep). The cells were then stained with different concentrations, ranging from 0.97 mM-10 mM, of cell trace violet (CTV, Lot # 1753289, Eugene) for 5 minutes at room temperature and washed once in 10 ml of R-5. Cells from each tube were then equally split into up to 4 aliquots and labelled with 0.08 mM-2.7 mM of carboxy fluorescein succinimidyl ester (CFSE, Cat # C34554, Life Technologies) in a final volume of 1 ml of R-5 for 5 minutes at room temperature. After washing the cells once, the cells were pulsed with 0.001-10 µg/ml of MHC-I or MHC-II binding peptide epitopes for 4 hours at 37°C. The cells were sedimented and washed with R-5 and pooled into two 2 ml aliquots. Pooled cells were labelled with either 2.7 mM or10 mM cell proliferation dye (CPD, Lot # E15450-106, eBioscience Inc.).

### 2.2.9.9.2 Peptide formulations
To assess NS5B-specific T cell responses NS5B peptides were used to stimulate splenocytes collected from naïve mice. The peptides were obtained through BEI Resources, NIAID, NIH: Peptide Array, Hepatitis C virus, K3a/650, NS5B protein. The peptides were 14- to 19-mer with 11 or 12 amino acid overlaps. Individual peptides were dissolved in 50µL 100% DMSO to a stock concentration of 20 mg/ml. Individual peptides were then mixed into pools of 30 peptides and diluted with RPMI (1% Pen/Strep) to a final concentration of 100ug/ml.

### 2.2.10 Epitope prediction and selection
Peptide prediction and selection was performed by Dr. Preston Leung, at the Viral Immunology Systems Program (VISP), The Kirby Institute, UNSW Sydney. Peptide sequences ranging from 14 to 19 amino acids from the HCV gt3a NS5B protein were used as input data for epitope prediction using IEDB MHC Class I epitope prediction algorithm (available at [www.iedb.org](http://www.iedb.org)). H2-kD was selected as the parameter for HLA type and epitope prediction length was restricted to 9 amino acids. *IEDB Recommended* approach was the algorithm selected to perform the
prediction. The result of this prediction was downloaded in .csv format. These data were then put through an in-house script to match with epitopes that were experimentally validated with strong immune response. The epitopes which matched the experimentally validated epitopes and had high prediction score (scores closer to 0.0 indicates a better predicted epitope) were selected for tetramer synthesis.

2.2.10.1 Antibody labelling of cells
Murine lymphocytes isolated from the liver and spleen were stained with a cocktail of different antibodies. First the cells were stained with fixable viability dye (FV620, Cat # 564996, BD Bioscience) diluted 1:500 for 20 minutes at room temperature in the dark. Cells were then washed and stained with a NS5B tetramer-APC (Bimolecular Research Facility, The John Curtin School of Medical Research, Canberra) diluted 1:50 for 45 minutes in the dark. The cells were then washed twice with PBS and labelled with a cocktail of antibodies including anti-CD44 FITC (Cat # 103006, Biologened) diluted 1:50, ant-CD11a BV510 (Cat # 563669, BD Bioscience) diluted 1:50, anti-CXCR3 BV421 (Cat # 562937, BD Bioscience) diluted 1:100, anti-CD8a AF780 (Lot # 4299472, eBioscience Inc.) diluted, anti-CD69 PE-Cy7 (Cat # 552879, BD Bioscience) diluted 1:100 and anti-CD62L PE (Cat # 553151, BD Bioscience) diluted 1:100. The fluorochromes conjugated to the antibodies in the cocktail include: phycoerythrin (PE) Cyanine (Cy) 7 or PE-Cy7 (CD69), APC-efluor® 780 (CD8), PE (CD62L), Brilliant Violet 421 (CXCR3) and Brilliant Violet 510 (CD11a).

2.2.10.2 Flow cytometry
Following staining of lymphocytes isolated from the liver and spleen, cells were analysed immediately on a BD FACSCanto II Flow Cytometer and the results were analysed using FlowJo software. Autofluorescence from cells was controlled by including unstained cell control and dead cells were discriminated from live cells using a FV620 staining. Compensation controls were run for each of the fluorochromes to correct for spectral overlap. When necessary,
Fluorescence minus one (FMO) controls were run to control data spread due to multiple fluorochromes.

2.3 In vivo techniques

2.3.1 Mice
Mice used in vaccination experiments, described in Chapters 4 and 5, were female BALB/c mice bred in specific pathogen free conditions at the University of Adelaide Laboratory Animal Services and housed at the Queen Elizabeth Hospital animal facility in PC2 compliant conditions. The mice were aged 6-8 weeks and weighed at least 16 grams at the start of experiments. The number of mice in each group was 7. Mice were weighed once weekly and clinical records were taken every day. C57BL/6 mice were used to titrate rAAV-NS5B-2A-eGFP.

2.3.2 Anaesthetic
For all procedures, mice were anesthetized by isofluorane provided by the animal facility.

2.3.3 Intradermal (ID) vaccination
Intradermal (ID) injection was used to deliver plasmid DNA vaccines to the dermis of the mice. Mice were anaesthetized, and the ear was swabbed with alcohol. An insulin syringe was used to inject the vaccine in a volume of 40 μl PBS; 20μl was delivered to each ear. The needle was inserted bevel side up so that the needle head was visible beneath the skin and vaccine was injected to form a bleb. The ears were observed for any inflammation or tissue damage post vaccination.

2.3.4 Intravenous (IV) tail vein injection
The mice were restrained and the tail vein was dilated by warming the tail with a heat lamp. The mice were injected with rAAV-NS5B-2A-GFP or PBS (in case of control groups) or FTA cells in a final volume of 200ul using an insulin syringe.
2.3.5 *In vivo transduction of mouse hepatocytes*

*In vivo* transduction of mouse hepatocytes with rAAV-NS5B-2A-eGFP was conducted by Dr. Kieran English at David Bowen’s laboratory, the Centenary Institute Sydney. Briefly, 8 weeks old male C57BL/6 mice were IV injected with different doses of rAAV-NS5B-2A-GFP or PBS. Seven days post injection hepatocytes were isolated by retrograde perfusion of the liver through the inferior vena cava using 25mL Hank’s balanced salt solution (HBSS). Infusion was started using HBSS supplemented with 0.5 mM EDTA followed by HBSS and completed using HBSS supplemented with 5mM CaCl and 0.05% Collagenase IV.

Following perfusion, the gall bladder was removed and discarded, the liver was removed and placed in a petri dish containing RPMI supplemented with 2% FCS where hepatocytes were separated from the liver by scraping with the back of a scalpel blade. The hepatocyte solution was then placed into a 50 ml falcon tube, then centrifuged at 500rpm for 5 minutes to pellet the cells and the supernatant was discarded. The hepatocytes were resuspended in 10 ml PBS and 6 ml of isotonic Percoll was added. The Percoll/hepatocyte solution was then centrifuged at 2000rpm for 10 minutes (Percoll allows live hepatocytes to form a pellet while dead hepatocytes float). The supernatant was discarded, and the hepatocyte pellet was resuspended in 10 ml FACS wash and stained with DAPI for flow cytometry.

Flow cytometry was carried out using the FACS Canto II (Becton Dickinson, North Ryde, NSW, Australia), and data were acquired using BD FACSDiva software (Becton Dickinson, North Ryde, NSW, Australia). Flow cytometry data were analysed using Flowjo software (Tree Star Incorporates, Ashland, OR, USA) version 9.8.3 for Mac OSX 10.10.2.
2.3.6  Spleen and Liver collection

2.3.6.1  Spleen collection
The spleen was removed from the left side of the abdomen using forceps and passed through a 70 μm cell strainer (BD #352350) using a 2 ml syringe plunger as a pestle. The cells were washed in 20 ml R-5 media and resuspended in 500 μl red blood cell lysis buffer (Sigma #R7757) for 45 seconds at room temperature to deplete red blood cells. The cells were then washed twice in 20 ml of R-5, resuspended in 2 ml of R-10 and cell numbers and viability assessed by trypan blue staining. The cells were then stained and analysed by flow cytometry.

2.3.6.2  Liver collection
The liver was harvested for the analysis of lymphocytes, but the abundant hepatocytes needed to be removed. The liver was perfused with 20 ml of PBS via the portal vein before it was removed from the animals. After removal it was homogenized by chopping in HBSS (3% FCS, 1% Pen/strep) using surgical scissors and passed through 70 μm cell strainer. The cells were then pelleted by centrifugation at 400 x g for 10 minutes. The cells were resuspended in 30% isotonic Percoll in HBSS and centrifuged at 500 x g for 20 minutes with no brake to allow the hepatocytes to float while lymphocytes and red blood cells were sedimented. The top layer was carefully removed using a Pasteur pipette and the lymphocytes in the pellet were washed twice with HBSS (3% FCS). One ml of RBC lysis buffer was added to the lymphocytes and incubated for 5 minutes at room temperature. Following RBC depletion, the lymphocytes were washed in HBSS (3% FCS) and resuspended in R-10. The cells were counted and stained for flow cytometry analysis.

2.4  Sample size
Based on earlier works [286-288], the study is powered to detect a difference in groups of 135 units at the 5% alpha level with 80% statistical power. Assuming unequal variance –standards deviations were found to be 38 and 100 units –a sample of 7 animals per group is required.
2.5 Statistical analyses

GraphPad Prism 6 software was used to construct the graphs presented in this study. All statistical analyses were performed using the IBM SPSS Statistics software (version 24). Data distribution were checked for normality using the Shapiro-Wilk test and normally distributed data were subjected to a parametric Levene’s test to test for homogeneity of variance. For normally distributed and homoscedastic data, one-way ANOVA with Tukey’s multiple comparison test was used to determine the statistical significance between comparisons. For all heteroscedastic data sets, Welch ANOVA with Games-Howell post-hoc was used to determine the statistical significance between comparisons. For non-normally distributed data a Kruskal-Wallis H test was used to determine the statistical significance of the data.
3 Chapter 3: Production of rAAV encoding HCV NS5B and GFP

3.1 Introduction

rAAV vectors have gained increased popularity in the field of gene therapy and vaccine development. Safe clinical outcomes and availability of different viral serotypes coupled with relatively sustained gene expression made them efficient options [304, 505]. AAV-based vaccines were tested against different infectious diseases [506], including HIV [507, 508], HPV [509, 510] and influenza virus [511]. Previously, an AAV-based malaria vaccine was shown to induce long-term antigen-specific antibody responses but was not protective from P. yoelii infection [512]. However, more recently a rAAV-based vaccine boosted the immune response from radiation-attenuated sporozoites and resulted in effective immune clearance of P. berghei following sporozoite challenge [455]. As both malaria parasites and HCV affect the liver, this chapter aimed to investigate the role of rAAV-based vaccines against HCV. To achieve this aim, rAAV encoding HCV gt3a NS5B and GFP (rAAV-NS5B-2A-eGFP) was required.

Several different approaches can be used to produce rAAV including the development of HEK 293 suspension cell lines that can grow in serum-free conditions, the baculovirus expression vector system (BEVS) utilizing Spodoptera frugiperda (Sf9) cells and the dual recombinant HSV (rHSV) infection system [513]. These different approaches share common factors, requiring the AAV non-structural proteins, the AAV structural proteins, and a set of helper-virus gene products and cellular factors necessary for macromolecular synthesis [405]. Yield inconsistency from the BEV system due to passage-dependent loss of function in the baculovirus helper [400] and the labour intensive and time consuming nature of the rHSV system [514-516] made triple transfection of HEK293 T cells the most common method to produce rAAV.

The triple transfection method uses the AAV cis- and trans-plasmids (ITR-gene of interest-ITR), along with a helper plasmid (pXX6) providing the adenoviral gene products necessary for replication and packaging, and a plasmid that encodes the Rep and Cap genes of AAV [304, 517].
Following transient transfection of HEK 293 T cells, the nascent virus is generally purified by ultrahigh speed density gradient centrifugation that involves either CsCl or Iodixanol (Optiprep); the latter results in a high virus titre [498]. However, the ultrahigh speed density gradient centrifugation method is time consuming and labour intensive [518]. A simpler method of rAAV purification that does not require ultracentrifugation was described by Guo et al. 2012 [498]. Briefly, transfected cells were freeze-thawed to release the virus, the virus was precipitated using PEG/NaCl and cellular proteins were removed using chloroform [498].

Thus, the aim of this chapter was to synthesize rAAV-NS5B-2A-eGFP using triple transfection of HEK293 T cells followed by PEG/NaCl precipitation to purify the virus.

3.2 Aims
The main aim of this chapter was to prepare and purify rAAV encoding gt3a HCV NS5B

Specific aims of this chapter were to:

1. Prepare and purify rAAV-NS5B-2A-GFP
2. Determine the titre of the virus that can transduce 10-25% of mouse hepatocytes in vivo

3.3 Results

3.3.1 Prepare and purify rAAV-NS5B-2A-GFP
The production of rAAV-NS5B-2A-eGFP requires three plasmids; i) pAM2AA-NS5B-2A-eGFP (containing the genes of interest (NS5B-2A-eGFP) flanked by the ITRs of AAV-2), ii) p5E1VD2/8 (containing the cap gene from AAV-8 and the rep-gene from AAV-2) and iii) pXX6 (containing Ad genes E2a, E4Orf6, vaRNA).

3.3.1.1 Insertion of FMDV2A and gt3a HCV NS5B into pAM2AA-eGFP
The construction of pAM2AA-NS5B-2A-eGFP was generated by including a PCR product comprised of the FMDV 2A sequence (2A) immediately upstream of eGFP and is described below.
The genes for HCV gt3a NS5B and eGFP were inserted into pAM2AA (described in 2.2.1) to ensure that protein expression was driven by the hAAT promoter. To ensure expression of NS5B and eGFP as individual proteins the sequence which encodes the 2A protease was inserted between the genes that encode the two proteins.

The 55bp sequence that encodes 2A was added as an overhang to a forward primer that was specific to eGFP. PCR amplification of eGFP, from pAM2AA-eGFP, using this forward primer and a reverse primer specifically binding to eGFP resulted in 2A-eGFP. The vector was then digested with the restriction enzymes EcoRI and HindIII to remove eGFP and the resultant product was then treated with Antarctic phosphatase to avoid religation of the linearised DNA. The 2A-eGFP PCR product was ligated into pAM2AA as described in section 2.2.4 (Figure 3.1).

Following transformation of SURE2 super-competent E. coli, random colonies were screened using forward and reverse primers that bind to the hAAT promoter and eGFP. Introduction of the 2A sequence was confirmed by sequencing. In order to insert NS5B, the sequence was PCR amplified using primers that contained NotI and HindIII restriction enzyme sites in the forward and reverse primers respectively. The amplified NS5B product and pAM2AA-2A-eGFP were then digested with NotI and HindIII. The insert was ligated to the linearised, Antarctic phosphatase-treated pAM2AA-2A-GFP (Figure 3.2).

All the PCR and digested products were analysed by 0.8-1% agarose gel electrophoresis to confirm the DNA amplicons and to confirm that digested products were of the correct size before successive purification (Figure 3.3).
Figure 3.1: Introduction of the 2A sequence upstream of eGFP. Primers to amplify eGFP were designed by including an EcoRI and the 2A sequence overhang in the forward primer and a HindIII overhang in the reverse primer. PCR amplification using these primers resulted in 2A-eGFP. Both the PCR product and pAM2AA-eGFP were digested with EcoRI and HindIII restriction enzymes. The digested products were ligated overnight at 16°C in the presence of T4 ligase enzyme and SURE 2 E. coli cells were transformed with the ligation mix. Colonies were screened by colony PCR and confirmed by restriction enzyme digestion and sequencing.
Figure 3.2: NS5B cloning in pAM2AA-2A-eGFP. Primers to amplify NS5B were designed by including NotI and EcoRI restriction enzyme sites in the forward and reverse primers, respectively. Both the PCR product and pAM2AA-2A-eGFP were digested with NotI and EcoRI enzymes. Ligation, transformation and screening of colonies were performed as described above in the legend to figure 3.1.
Figure 3.3: Gel photographs taken in the process of cloning NS5B-2A into the pAM2AA-GFP vector. A) PCR amplification of eGFP by including 2A sequence as a forward primer overhang. B) Digestion of pAM2AA-eGFP to remove eGFP. C) Random colonies, tracks 1, 2 & 3, were picked from agar plates and screened with colony PCR to detect positive clones. D) Confirmatory digestion of pAM2AA-2A-eGFP mini-prep. (EcoRI single digest = 6297bp; HindIII/EcoRI double digest = 5514bp + 783bp) E) PCR amplified product of HCV gt3a NS5B. F) Digestion of pAM2AA-2A-eGFP using Not I/EcoRI double digestion. G) Random colonies, tracks 1, 2 & 3, were picked from agar plates and screened by colony PCR to detect positive clone. H) Confirmatory digestion of pAM2AA-NS5B-2A-eGFP mini-prep (BamHI single digestion= 8041; KpnI/EcoRI double digestion = 2871bp +5170bp). ( * indicates expected band sizes).
3.3.1.2 Protein expression from pAM2AA-NS5B-2A-eGFP

3.3.1.2.1 eGFP expression

The above sections reported the construction of pAM2AA-NS5B-2A-eGFP but it is necessary to ensure that the encoded proteins were expressed. Initially, the level of eGFP expression was assessed in HEK293 T cells transfected with pAM2AA-NS5B-2A-eGFP. The transfected cells were incubated for 48 hours and then examined by fluorescence microscopy. No background fluorescence was detected in cells that were left untransfected as a negative control. The intensity of eGFP expression was lower than that of the positive control pVAX-eGFP most likely attributed to the strong CMV promoter in pVAX as compared to hAAT in the pAM2AA vector. It has been shown that the alpha-1 antitrypsin promoter was able to result in transgene expression in 40% of the cells relative to the CMV promoter [519]. However, the experiment confirmed that hAAT was capable of directing expression of the inserted sequences (Figure 3.4) from the construct.

Figure 3.4: eGFP expression in HEK 293 T cells 48 hours post transfection with pAM2AA-eGFP. HEK 293 T cells were transfected with 200μg of DNA using lipofectamine LTX plus reagent. Forty-eight hours post transfection the cells were examined for GFP expression. (Micrographs were taken using a ZEISS fluorescent microscope at 488μm wavelength and 10x magnification).
3.3.1.2.2 NS5B expression

Although expression of eGFP from the monocistronic pAM2AA-NS5B-2A-eGFP plasmid suggests that NS5B would also be expressed, it was considered necessary to confirm expression of NS5B directly. This was examined by immunofluorescence staining of HEK 293 T cells following transfection with pAM2AA-NS5B-2A-GFP. Forty-eight hours post transfection the cells were fixed with 4% paraformaldehyde (PFA), permeabilised and stained with HCV positive human serum as described in Chapter 2, section 2.2.8. As shown in Figure 3.5, as a polyclonal serum was used and a protein was detected, this was most likely to represent NS5B in pAM2AA-NS5B-eGFP transfected cells (Figure 3.5 A) while expression was not observed in un-transfected cells (Figure 3.5 B). Moreover, NS5B expression correlated with eGFP expression as shown in the overlay (Figure 3.5 A).

![Figure 3.5: Immunofluorescence staining to detect NS5B expression from pAM2AA-NS5B-2A-eGFP. HEK293 T cells were transfected with pAM2AA-NS5B-2A-eGFP (A) or left un-transfected as a control (B). 48 hours post transfection, the cells were fixed, permeabilised and immunofluorescence was performed as described in Chapter 2, section 2.2.8. (Micrographs were taken using a ZEISS LSM 700 fluorescent microscope at 10x magnification).](image-url)
3.3.1.3 Production of rAAV encoding HCV gt3a NS5B and eGFP

Transfection of HEK 293 T cells with the three plasmids, pAM2AA-NS5B-2A-eGFP, p5E1VD2/8 and pXX6, will result in the synthesis of rAAV-NS5B-2A-eGFP. The three plasmids were added at an equimolar ratio and mixed with PEI at a 3:1 ratio of PEI (1mg/ml) to total DNA as described in Chapter 2 section 2.2.9.3. The transfection efficiency was monitored by microscopic examination of eGFP expression (Figure 3.6). The transfected cells were incubated for 72 hours before virus harvest and purification as described in Chapter 2, section 2.2.9.4.

![Figure 3.6: Monitoring eGFP expression following triple transfection.](image)

Figure 3.6: Monitoring eGFP expression following triple transfection. HEK293 T cells were transfected with the three plasmids using PEI to synthesise the rAAV-NS5B-2A-eGFP virus. Transfection was monitored using microscopic examination of eGFP expression at 24-, 48- and 72-hours post transfection. (Micrographs were taken using a ZEISS fluorescent microscope at 488nm wavelength and 10x magnification).

3.3.1.4 rAAV characterization.

In purified rAAV stocks the dominant proteins that should be present are the three viral capsid proteins; VP1, VP2 and VP3 [306]. The molar ratio of VP1, VP2 and VP3 in the rAAV particle
is 1:1:10 and their molecular mass is 87, 72 and 62kDa, respectively [298]. SDS-PAGE is used to determine the capsid composition of the purified virus [306].

SDS-PAGE was performed as described in Chapter 2 section 2.2.9.5 using 5 µl of concentrated virus. As depicted in figure 3.7, the three VP of the capsid VP1, VP2, and VP3 were readily detected. The thick intense band with a molecular weight between 50 and 75kDa represents VP3 (Figure 3.7). These results were comparable those reported by Guo et al., who employed a similar purification technique [498]. Due to the high cost of anti-capsid antibodies and because subsequent in vitro studies (section 3.3.1.6) revealed expression of eGFP and NS5B (see below), western blotting of the virus capsid was not performed.

Figure 3.7: Coomassie blue stained SDS-PAGE of denatured rAAV. (rAAV-GFP-N4OVA, purified in the same manner, was used as a positive control (The pAM2AA-GFP-N4OVA plasmid used to synthesize rAAV-eGFP-N4OVA was a kind gift from David Bowen, Centenary Institute, University of Sydney)).
Once the viral capsid proteins were detected, it was necessary to determine that all the encoded genes were packaged. The purified virus was treated with DNase, the virus capsid was lysed to release the viral genome using phenol chloroform [520] and the extracted genome was used as a template for a PCR. The primers used for amplification encompassed the recombinant virus genome region from the hAAT promoter to the end of eGFP sequence (Figure 3.8 A). PCR was performed as described in chapter 2 (section 2.2.2.) and the products were analysed by 1% agarose gel electrophoresis to confirm the size of the amplicons. As a positive control, the PCR was performed using pAM2AA-NS5B-2A-eGFP plasmid DNA as a template. The results showed amplicons with expected band sizes that were similar between the PCR products from the virus genome and the plasmid DNA (Figure 3.8 B).

Figure 3.8: PCR amplification of NS5B-2A-eGFP sequences from DNA extracted from rAAV-NS5B-2A-eGFP. A) The location of primers used for the PCR. B) Analysis of the PCR products by 1% agarose gel electrophoresis.

3.3.1.5 Titration of rAAV-NS5B-2A-eGFP by quantitative PCR
The titre of rAAV-NS5B-2A-eGFP was determined using a commercial kit (AAV pro-titration kit for real time PCR V.2) as described in section 2.2.9.6. The viral genome was released from
purified virus as described above, diluted as necessary and used as a template for quantitative PCR. The PCR was performed using the conditions described in Section 2.2.9.6

The cycling melt curve and standard curve are shown in figure (Figure 3.9). The titre of the virus was determined based on the standard curve produced from the positive control provided in the kit. The positive control is a plasmid DNA that includes the AAV ITRs, adjusted to $2 \times 10^7$ copies/μl based on the value at OD$_{260}$. The plasmid was serially diluted, ranging from $2 \times 10^2$ to $2 \times 10^7$ copies/μl, to produce a standard curve for viral particle quantification. Based this standard curve, the average titre of virus obtained was $8.5 \times 10^8$ vgc/μl and the total yield from the transfection of 40 T-75 flasks was $2.5 \times 10^{11}$ vgc.

Figure 3.9: Representative images taken from the titration of rAAV-NS5B-2A-eGFP using quantitative PCR. DNAse treated, lysed virus was diluted using EASY dilution buffer (1:50) and 5μl of the diluted sample was used as a template for qPCR.

3.3.1.6 Transduction of HT1080 cells by rAAV-NS5B-2A-eGFP

The next logical step to assess successful construction of the virus was to check protein expression from the encoded genes. For this, HT1080 cells were transduced with rAAV-NS5B-2A-eGFP to examine expression of NS5B and eGFP. HT1080 cells are human fibrosarcoma cells that are
shown to be readily transduced by rAAV [521]. The cells were seeded at a density of 2.5 x10^4 cells/well in a 96 well cell culture plate and rAAV-NS5B-2A-eGFP was added to the cells at the multiplicity of infection (MOI) of 10^5. As a positive control for eGFP expression, cells were transduced with rAAV-eGFP-N4OVA at a similar MOI. eGFP expression was assessed by direct microscopic observation and flow cytometry analysis while immunofluorescence staining was used to detect NS5B expression.

Direct microscopic examination of transduced cells revealed expression of eGFP from the virus (Figure 3.10 A). Flow cytometry analysis showed only 3% and 12% of the cells transduced with rAAV-NS5B-2A-eGFP and rAAV-eGFP-N4OVA, respectively, were eGFP positive (Figure 3.10 B). Although the percentage of eGFP positive cells confirmed eGFP expression from rAAV-NS5B-2A-eGFP, it was difficult to detect NS5B expression using immunofluorescence staining most likely due to the low level of transduction. Hence, it was necessary to improve the level of transduction. To achieve this 5µM Hoechst 33342 (Invitrogen) was used as a transduction enhancer. It was reported that Hoechst 33342 enhances transduction of different cell lines by rAAV [501]. Accordingly, the level of transduction was improved from 3% to 17.8% in case of rAAV-NS5B-eGFP and from 12% to 28% in the case of rAAV-eGFP-N4OVA (Figure 3.10 B). Other studies reported greater than 600 GFP-positive cells per field when a similar MOI was used to transduce HT1080 cells in the presence of 10 µM Hoechst 33342 [502]. Others used a MOI of 10^4 to transduce HEK293 T cells, but fluorescent intensity of eGFP, not the frequency or percentage of transduced cells, was reported [522].

Using Hoechst 33342 as a transduction enhancer, expression of NS5B was assessed at two different stages. First using RT-PCR, transcription of the encoded genes was checked at the RNA level. RNA was extracted from the transduced cells using the RNeasy Mini-kit according to the manufacturer’s instructions and cDNA was synthesised using the QuantiTect reverse transcription kit. This cDNA was used as a PCR template for RT-PCR and amplified using primer
pairs that bind specifically to the NS5B-2A-eGFP region. The primers (See Appendix IX for the list of primers) were designed in such way that the amplicon size was <400bp (Figure 3.11 A). A small amplicon was selected because the efficiency of amplification decreases as the amplicon size increases [523]. The housekeeping gene, GAPDH, was used as a control for reverse transcription.

**Figure 3.10: eGFP expression following transduction of HT1080 cells with rAAV-NS5B-2A-eGFP or rAAV-eGP-N4OVA.** HT1080 cells were transduced with rAAV at a MOI of 10⁵ in the presence of 5µM Hoechst 33342. Seventy-two hours post transduction representative images of eGFP expression were taken using a Zeiss microscope (A) and the percentage of cells expressing eGFP was determined using flow cytometry (B)
The RT-PCR was performed in a 3-step cycling program with initial denaturation at 95°C for 2 minutes, followed by 35 cycles at 95 °C for 5 seconds and fluorescent detection at 60 °C for 30 seconds. This showed that all the expected products were detected revealing that mRNA was transcribed from the virus genome (Figure 3.11 B).

Figure 3.11: RT-PCR to detect NS5B and eGFP RNA in HT1080 cells transduced with rAAV-NS5B-2A-GFP. RNA was extracted from transduced cells and cDNA was synthesised which was then used as a template for RT-PCR. A) Primers designed to amplify cDNA fragments. B) RT-PCR showing amplification of segments of the cDNA using primers made in A.

Next NS5B expression was confirmed using immunofluorescence staining. HT1080 cells were transduced as described above and 72 hours post transduction, the cells were fixed and stained using HCV-positive human serum as a source for the primary antibody and goat anti-human
AlexaFluor-555 as a secondary antibody. Microscopic examination of stained cells revealed expression of NS5B and eGFP in rAAV-NS5B-2A-eGFP transduced cells (Figure 3.12 A). Although a few cells appeared to express NS5B but not eGFP, also reported by others [524], in general the two proteins were colocalised. In cells transfected with pVAX-GFP only GFP expression was observed (Figure 3.12 B) while un-transfected cells showed no expression of eGFP or NS5B (Figure 3.12 C) confirming that the NS5B expression observed in rAAV-NS5B-2A-eGFP transduced cells was specific (Figure 3.12 A).

**Figure 3.12: Immunofluorescence staining to detect NS5B expression from rAAV-NS5B-2A-eGFP.** HT1080 cells were transduced with $10^5$ vgc of rAAV-NS5B-2A-eGFP (A) or transfected with pVAX-GFP (B) or left un-transfected (C). Seventy-two hours immunofluorescence was performed as described in Chapter 2, section 2.2.8.
3.3.1.5 Transduction of mouse hepatocytes in vivo by rAAV-NS5B-2A-eGFP

A previous study showed that a dose of rAAV capable of transducing 5-10% of hepatocytes in vivo was highly immunogenic, but a dose which resulted in >25% of transduction of hepatocytes was tolerogenic [382]. To determine the dose of virus needed to transduce 10-25% of mouse hepatocytes, mice were injected IV with rAAV-NS5B-2A-eGFP or PBS in the case of the control mouse. Mice received different doses of the virus; 2x10⁹, 5x10⁹, 2x10¹⁰, 5x10¹⁰ or 2x10¹¹ vgc. Seven days after injection, the mice were euthanised and liver samples were harvested. Hepatocytes were isolated according to the procedure described in section 2.3.5. The cells were stained by DAPI and analysed by flow cytometry to determine the percentage of GFP-expressing hepatocytes. From this analysis, a virus dose of 5x10⁹ vgc resulted in 10% transduction of mouse hepatocytes (Figure 3.13) and this dose was used for subsequent experiments. In vivo transduction and analysis of mouse hepatocytes was performed by Dr. Kieran English at the Centenary Institute, Sydney.

3.4 Discussion

The use of rAAV in gene therapy and to deliver vaccine antigens has been described previously [373, 525-527]. Therefore, the aim of this chapter was to synthesize rAAV-NS5B-2A-eGFP that can be used as a trapping agent for HCV-specific CD8 T cells in the liver, that are induced following vaccination with pVAX-NS5B-PRF.

The production of rAAV requires three plasmids; the plasmid that contains the gene of interest flanked between the ITRs, the plasmid that encodes the rep and cap genes and the helper plasmid that contains the adenoviral helper genes [497]. The first section of this chapter described the construction of the plasmid that encodes HCV gt3a NS5B and eGFP, pAM2AA-NS5B-2A-eGFP.
Figure 3.13: Analysis of eGFP expression in isolated mouse hepatocytes. The percentage of eGFP positive mouse hepatocytes was assessed seven days post injection with different doses of rAAV-NS5B-2A-eGFP. Eight weeks old male C57BL/6 mice were IV injected with different doses of rAAV-NS5B-2A-eGFP. Seven days later hepatocytes were harvested and eGFP expression was analysed by flow cytometry.

The pAM2AA vector was previously described and contains a liver specific, ApoE/hAAT enhancer/promoter [382, 528]. This restricts expression of the encoded genes to the liver [528]. Following successful cloning of NS5B and eGFP, these proteins need to be expressed as individual proteins from a single promoter. To achieve this the 2A protease was designed to cleave eGFP from NS5B [206, 529]. Expression of eGFP and NS5B from pAM2AA-NS5B-2A-eGFP was demonstrated following transfection of HEK293 T cells.

In the second section of this chapter, production of rAAV-NS5B-2A-eGFP was described. The virus construct was packaged in HEK 293 T cells with the help of the pXX6 plasmid and
pseudotyped to AAV type 8 capsid because this serotype was shown to increase transduction of hepatocytes [530, 531]. It has been reported that a lower proportion of hepatocytes are permissive to rAAV-2 and increasing the vector dose failed to show a linear relationship with the number of transduced cells and the level of transgene expression [532]. On the other hand, rAAV-8 showed a linear dose-response curve at a dose as high as $7.2 \times 10^{12}$ vgc, making it the preferred serotype to transduce hepatocytes [530].

The ultra-high-speed density gradient centrifugation technique to purify rAAV is regarded as labour intensive and time consuming with significant amount of vector loss [518]. As a result, a simpler method of purification that avoids ultra-high-speed centrifugation was introduced by Guo et al., 2012 [498]. Using this technique $6.25 \times 10^8$ vgc/ul of rAAV-NS5B-2A-eGFP was obtained as titrated by AAV-Pro titration kit (for real time PCR) Ver.2. The purified virus was also tested for its ability to transduce HT1080 cells in vitro and mouse hepatocytes in vivo.

In vitro transduction of HT1080 cells, at a MOI of $10^5$, with the purified rAAV-NS5B-2A-eGFP virus resulted in approximately 18% GFP positive cells by flow cytometry. The use of DNA minor groove binding agents Hoechst 33258 and 33342 was reported to increase transgene expression from rAAV [501]. Similarly, in this experiment the use of 5uM Hoechst 33342 improved eGFP expression from 3% to 18% from rAAV-NS5B-2A-eGFP while it increased eGFP expression from rAAV-GFP-N4OVA from 13% to 28%. Although the effect of Hoechst is not clearly understood, it was suggested that the compound has a significant effect on the different stages of the cell cycle [501]. Although, the expression of eGFP can be considered as an indicator of NS5B expression because the expression of both proteins was driven by a single promoter and the 2A protease ensures cleavage of the two proteins as described elsewhere [206, 529], it was equally important to detect NS5B expression. Accordingly following transduction of HT1080 cells NS5B-specific mRNA was detected by RT-PCR and immunofluorescence staining confirmed expression of NS5B.
From a previous study it was indicated that the proportion of antigen-positive hepatocytes resulting from in vivo transduction with rAAV determines the fate of the CD8⁺ T cell response in the liver [382]. According to this study, transduction of a high percentage (>25%) of hepatocytes will result in exhaustion of CD8⁺ T cells [382]. Hence, rAAV-NS5B-2A-eGFP was titrated to determine the dose of virus required to transduce 10-25% of hepatocytes. From this experiment 5x10⁹ vgc of rAAV-NS5B-2A-eGFP was able to transduce 10.6% of mouse hepatocytes, in vivo, and this dose was selected as an immunologically functional virus dose for subsequent experiments.

3.5 Conclusion
The results in this chapter report cloning of HCV gt3a NS5B and eGFP under a liver specific promoter in a plasmid, pAM2AA-NS5B-2A-eGFP, that was later used to produce a rAAV that encodes these proteins. The data showed that rAAV-NS5B-2A-eGFP was able to transduce HT1080 cells in vitro and both eGFP and NS5B were detected in these cells. It was also shown that rAAV-NS5B-2A-eGFP was able to transduce mouse hepatocytes in vivo.
4 Chapter 4: *In vivo* T cell epitope mapping of gt3a HCV NS5B

4.1 Introduction

A recent study from our laboratory that compared CMI to NS3, NS4 and NS5B from gt1b and gt3a HCV in DNA-vaccinated BALB/c mice showed that the highest magnitude of T helper and CD8+ killer T cell responses were directed against gt3a NS5B *in vivo* [288]. Thus, gt3a NS5B was chosen as a model antigen to optimise a vaccination strategy to elicit HCV-specific CMI in the liver.

Seminal studies that described the biological roles of CD8+ T<sub>RM</sub> cells stimulated adoptively transferred CD8+ T cells that express a unique congeneric marker on the cell surface compared to endogenous CD8+ T cells found in recipient mice [455, 495]. In the context of vaccination, it is more physiological to analyse endogenous antigen-specific CD8+ T cells and a convenient way to achieve this *ex vivo* is to use tetramers, which are essentially synthetic peptide-MHC-I monomers, joined by biotinylated streptavidin, conjugated to a fluorochrome [533, 534]. It is also important to analyse HCV-specific CD4+ T helper cell responses as NS3-, NS4- and NS5-specific CD4+ T cell responses also correlate with natural recovery from acute HCV infection [152]. However, a reliable method to develop successful tetramers to analyse antigen-specific polyclonal CD4+ T cells that develop *in vivo* is not available. Thus, to identify the immunodominant T cell epitopes of gt3a NS5B *in vivo*, an in-house FTA technology was exploited. The FTA is the most versatile assay to measure the breadth, avidity, magnitude and epitope variant cross-reactivity of CD8+ killer T cell and CD4+ T<sub>H</sub> cell responses *in vivo* in mice [503, 535]. This technology will also be used to analyse T cell responses in the spleen and the liver *in vivo* which is important given that T<sub>RM</sub> cells, including those recovered from the liver, survive poorly in functional assays performed *in vitro* [433, 455].
This chapter describes the FTA-based mapping process of immunodominant T cell epitopes of gt3a NS5B in the liver and the spleen of BALB/c mice. In the FTA technology, CD8\(^+\) T cell responses are measured by target cell (cells loaded with peptides representing the vaccine antigen) killing while CD4\(^+\) T cell responses are measured by the upregulation of CD69 on FTA B (B220\(^+\)) cells [504, 535]. As noted in section 1.3.2.1, CD69 is associated with specific tissue residency, but CD69 is also recognized as a type II C-type lectin involved in lymphocyte migration and cytokine secretion [536] and is an early indicator of leucocyte activation [536, 537]. MHC-II-restricted antigen presentation by peptide-loaded B cells will result in an antigen-specific interaction with T\(_H\) cells. This interaction leads to activation and differentiation of the B cells resulting in high levels of expression of CD69, and this can be used to measure the CD4\(^+\) T cell helper response resulting from vaccination [538, 539].

4.2 Aims

The aims of this chapter are to:

1. Map the immunodominant T cell epitopes of gt3a NS5B following vaccination of BALB/c mice with pVAX-NS5B-PRF

2. Synthesize and test a MHC-I tetramer (H2-K\(^d\)) in BALB/c mice based on the most immunodominant peptide mapped for CD8\(^+\) killer T cell responses.

4.3 Results

4.3.1 Immunodominant peptides of HCV gt3a NS5B recognised by T cells

- Naive female BALB/c mice (7 mice per group) were immunised on two occasions at fortnightly intervals with 50 \(\mu\)g of pVAX-NS5B-PRF or pVAX-PRF using equimolar amounts of DNA.

Thirteen days after the final DNA immunisation, the mice were challenged IV with autologous fluorescent, bar-coded splenocytes loaded with each of the NS5B peptides spanning NS5B\(_{385-591}\) (pool 3 (P3) as described in the Methods chapter (2.2.10.1). NS5B\(_{385-591}\) was chosen given the
published data from our laboratory which showed that this region contained the most immunodominant CD8\(^+\) cytotoxic T cell epitopes [288]. The specific FTA matrix and the bar-coding scheme used in this chapter are shown in the diagram below (Figure 4.1). Fifteen hours post FTA challenge, the mice were euthanised and the FTA cells recovered from the spleen and the liver were analysed using flow cytometry (Figure 4.2). Each peptide was tested only once. However, peptides identified to contain CD8 and CD4 T cell epitopes were tested twice.

![Diagram of FTA setup](image)

**Figure 4.1: FTA setup for the identification of T cell epitopes in HCV gt3a NS5B P3.** The numbers in brackets correspond to the individual peptides in P3 of HCV gt3a NS5B. Nil; these cells were not loaded with any peptide. P3: targets pulsed with the pooled peptides of gt3a NS5B.

Following flow cytometric analysis, the CTL response was measured based on the percentage of specific FTA lost (% specific killing) that was calculated according to the following formula described by Wijesundara et al., [503].

$$\text{% specific killing} = \left( \frac{\text{% of nil targets} - \text{% of peptide pulsed target}}{\text{% of nil targets}} \right) \times 100$$
Figure 4.2: Representative plot of analysis of FTA cells recovered from the spleen of mice vaccinated with pVAX-NS5B-PRF. HCV gt3a NS5B-specific CTL responses were examined *in vivo* following vaccination of mice with pVAX-NS5B-PRF or pVAX-PRF. Female BALB/c mice (n = 7 per group) were vaccinated and 13 days after the final vaccination, $2 \times 10^6$ peptide-loaded cells (for each target cell cluster) or mock target cells were transferred IV. Fifteen hours after FTA challenge, splenocytes and liver lymphocytes were harvested and analysed by flow cytometry to determine the surviving number of peptide-pulsed target cells relative to unstimulated target cells. (A) Gating strategy to discriminate lymphocytes. Lymphocytes were double discriminated using SSC-A/FSC-A followed by SSC-W/SSC-H and FSC-W/FSC-H before analysis of targets. B) Analysis of FTA cell death. All the peptide pulsed cell targets were gated and analysed for the percentage recovery relative to mock targets to determine the specific FTA cell loss. The cells gated using CFSE/CTV correspond to the table on the top which in turn is related to the scheme indicated in Figure 4.1.
The percentage loss of FTA target cells loaded with peptide 69 (P69; NS5B$_{442-459}$; PLDFEMYGATYSVTPLDL) or peptide 70 (P70; NS5B$_{449-466}$; GATYSVTPLDLPAIERL) was comparable to the percentage loss of targets pulsed with P3 (Figure 4.3). The concentration of an individual peptide when used alone and its concentration when used in the pool was constant, \textit{i.e.}, 10\,\mu g/ml. In the spleen, 84.5\% and 65.1\% of targets loaded with P69 and P70 were lost, respectively, while the corresponding percentage losses in the liver were 64.5\% and 47.2\% (Figure 4.3).

**Figure 4.3:** Specific loss of FTA cells pulsed with peptides in pool 3 of HCV g3a NS5B. FTA cells were loaded with 10\,\mu g/ml of either individual peptides in P3, the combined P3 or mock, and transferred to vaccinated mice (n= 7 mice per group). Fifteen hours after transfer spleen and liver were harvested and lymphocytes were isolated as described in Materials and Methods (section 2.3.6). Surviving peptide-loaded cells recovered from the liver and spleen were analysed by flow
cytometry and the specific loss of peptide FTA cells was calculated and plotted using Graph pad prism software.

Overall the *in vivo* T cell mapping study using the FTA assay revealed two immunogenic peptides, P69 and P70, that were likely to be targets of CD8⁺ T cells.

Peptides in P3 were also analysed for the presence of CD4⁺ T cell epitopes (Figure 4.4). Following the analysis of CD69 expression on recovered FTA B cells, the geometric mean fluorescent intensity (GMFI) of CD69 was determined. Expression of CD69 was calculated by subtracting the GMFI of CD69 in naive mice from that in the vaccinated mice [503]. Accordingly, peptide 77 (P77; NS5B₄⁹⁵-₅¹₂) was identified as a target to increase expression of CD69 in the spleen, but not in the liver, of vaccinated mice. None of the remaining peptides was considered as a T₉ cell immunodominant epitope as the GMFI of CD69 was not significantly higher in vaccinated mice compared with unvaccinated (mock) mice (Figure 4.4).

On the other hand, recently published data from our laboratory showed that pool 2 (P2: NS5B₁₉₇-₃₉⁵) contains immunodominant peptides that are targets for CD4⁺ T cells [288]. As a result, NS5B peptides in P2 were assessed to determine any additional immunodominant peptides responsible for a helper T cell response. The results showed that a relatively higher GMFI of CD69 expression was observed in the spleen in FTA cells that were pulsed with peptide 36 (P36: NS5B₂₂₅-₂₄₀) while responses in the liver were not significantly higher than the mock (Figure 4.5 A). From this study it was concluded that P36 was an immunodominant T₉ cell epitope in the entire P2 of HCV gt3a NS5B. Overall, peptide 77 (NS5B₄⁹⁵-₅₁₂; PPLRAWRHRARAVRAKLI) from P3 and P36 (NS5B₂₂₅-₂₄₀; DSTVTGQDIRVEEAVY) from P2 were identified to be immunodominant peptides for a CD4⁺ T cell response.
To confirm the previously reported data on the absence of CTL epitopes in P2 of NS5B, the level of specific killing of FTA targets loaded with P2 peptides was analysed (Figure 4.5 B). The analysis showed that none of the peptides in P2 was a target of a CTL response (Figure 4.5 B).

Negative percentages following FTA analysis are indicative of no significant killing of peptide loaded target cells above that of the respective mock-pulsed target cells injected into animals. Similarly, negative GMFI results indicate the absence of a significant CD69 expression on FTA B220⁺ target cells that were loaded with peptides as compared to mock-loaded target cells injected into animals.

4.3.2 Synthesis of MHC-I tetramer to characterize CD8⁺ T cells
Tetramers have emerged as a key tool to determine the quantity and various phenotypic characteristics of antigen-specific CD8⁺ T cells. They can be combined with other staining techniques to derive detailed information on antigen-specific T cells, such as activation, effector function, proliferation and apoptosis [534, 540, 541]. Tetramers are designed and synthesised from immunogenic peptides by selecting the most immunodominant sequences comprised of nine amino acids [534, 542, 543]. In this section, the selection of the nine amino acid sequence and the synthesis of the tetramer using MHC-I (H2-Kd in BALB/c mice) is described.
Figure 4.4: CD69 expression on B220$^+$ FTA cells. FTA cells were loaded with 10$\mu$g/ml of either individual peptides in P3, P3 or mock, and transferred to vaccinated mice. Fifteen hours later spleen and liver were harvested, and surviving lymphocytes were isolated and analysed by flow cytometry. The GMFI of CD69 expression on B220$^+$ cells was calculated using excel spread sheet and plotted using Graph pad prism software. A) Representative plot of gating strategy for FTA B220$^+$ cells. Lymphocytes were double discriminated as in Figure 4.2 followed by gating on B220$^+$ cells. B) GMFI of CD69 as plotted using Graph pad prism software.
**Figure 4.5:** FTA analysis to identify immunodominant epitopes in P2 of HCV gt3a NS5B.

FTA cells were recovered and analysed using flow cytometry and double discriminated as shown in Figure 4.2. CD69 expression on B220+ FTA cells recovered from the liver and spleen were plotted as GMFI of CD69 (A). Similarly, the percentage loss of FTA cells was determined according to the formula described above and depicted (B).
The CTL immunodominant peptides, P69 and P70, identified from P3 of HCV gt3a NS5B were used as input to identify the most immunodominant nine amino acid sequence. Preston Leung from the Kirby Institute, University of New South Wales, used bioinformatics to predict the most immunogenic nine amino acid sequence. Based on the algorithm described in section 2.10.2, NS5B\textsubscript{451-459} (TYSVTPLDL), was identified as the most immunodominant region recognised by CD8\textsuperscript{+} T cells. To confirm that this sequence is responsible for the observed CTL response, a peptide (immunodominant, ID) with this sequence was synthesised (China peptides, China) and loaded on to FTA target cells. FTA target cells were then transferred to vaccinated mice and the percentage killing response was determined (Figure 4.6). As depicted in the figure, the percentage loss of FTA target cells loaded with ID was similar to those in targets loaded with P69, P70 or P3. All the peptides as well as the ID peptide were used at 10\(\mu\text{g/ml}\) concentration. The level of the killing response was also similar in the spleen and in the liver.

![Figure 4.6: Percentage specific loss of peptide pulsed FTA cells.](image)

FTA cells were pulsed with P69 (NS5B\textsubscript{442-459}), P70 (NS5B\textsubscript{449-466}), NS5B\textsubscript{451-459} or P3 (NS5B\textsubscript{385-591}) of NS5B. Peptide loaded FTA cells were transferred to vaccinated or unvaccinated mice (n= 7mice per group) and analysed 15 hours later as described above. The percentage specific killing response was assessed in the liver and spleen of mice.
The next step was to synthesize a MHC-class I binding tetramer based on the NS5B_{451-459} peptide. The tetramer was synthesized based on the murine H2-K^{d} MHC-I allele and labelled with allophycocyanin (APC) at the ACRF Biomolecular Resource Facility, John Curtin School of Medical Research, Australian National University, Canberra. Before using this tetramer in large scale experiments, it was also tested in a pilot experiment in which mice (7 mice in each group) were vaccinated with two doses of 50 μg of either pVAX-NS5B-PRF or pVAX-PRF two weeks apart. Seven days after the second vaccination splenocytes were collected, stained with the tetramer and analyzed by flow cytometry. Based on the results from this pilot experiment, the tetramer was able to specifically stain NS5B specific CD8^{+} T cells. Accordingly, a significantly higher (P < 0.0001) number of tetramer positive NS5B specific CD8^{+} T cells were identified in the pVAX-NS5B-PRF vaccinated mice (Figure 4.7).

**Figure 4.7: Tetramer analysis of NS5B specific CD8^{+} T cells.** Mice were vaccinated with pVAX-NS5B-PRF or pVAX-PRF and isolated splenocytes were stained with APC-K^{d}NS5B_{451-459} for 45 minutes at room temperature. A) Representative plot of tetramer positive CD8^{+} T cells. B) Comparison of the number of tetramer positive CD8^{+} T cells between pVAX-NS5B-PRF and pVAX-PRF vaccinated groups (n= 7 per group).

### 4.4 Discussion

Quantitative parameters are important in understanding the dynamics of a T cell immune response following vaccination [533, 544]. The quantitative analysis of antigen-specific T cell responses has been significantly advanced by the development of MHC-peptide complexes [533]. While
tetramers provide valuable information regarding the number of antigen-specific cells, they fail to provide information about the functionality of these cells [534, 545] and consequently, different methods have been developed to address this limitation [544, 546-550]. *In vivo* CTL killing assays [544, 548, 550, 551] are used to measure the magnitude of the killing response, although, they cannot determine details such as functional avidity. *In vitro* assays like cytokine staining assays and ELISpot peptide dilution assays require *ex vivo* stimulation of host effector T cells which may result in changes in the functional avidity due to a preferential outgrowth of high avidity effector T cells [161]. On the other hand, the FTA technology can provide detailed measurement on multiple parameters of T cell responses in a single animal, *in vivo* [503, 504, 535]. Hence the main aim of this chapter was to determine the immunodominant epitopes of HCV gt3a NS5B using FTA technology and to develop a MHC-peptide tetramer for the analysis of NS5B-specific CTL.

Initially it was necessary to identify NS5B peptides recognised by CD8\(^{+}\)- and CD4\(^{+}\)- T cells. The mapping process basically involved the transfer of fluorescently labelled, peptide loaded naïve splenocytes to mice vaccinated with either pVAX-NS5B-PRF or pVAX-PRF as described in Chapter 2 section 2.2.10 [503, 504]. CD8\(^{+}\) T cell epitopes were identified based on the specific killing or loss of FTA target cells. The efficiency of the killing response was based on the relative ratio of target cells recovered from vaccinated mice compared to the ratio in unvaccinated mice [535, 552]. From a previous report it was noted that pool 3 of NS5B was the immunodominant region that was targeted by CD8\(^{+}\) T cells [288]. Accordingly, individual peptides from P3 were loaded onto FTA cells to identify the most immunodominant peptide. From flow cytometry analysis it was evident that the loss of FTA cells loaded with P3 was similar to the published data [288]. More specific analysis determined that P69 and P70 were the main targets for CTLs. Particularly, the loss of target cells pulsed with P69 was comparable to the loss of target cells pulsed with the entire P3 peptides both in the liver and the spleen of vaccinated mice. CTL killer
responses of cells pulsed with P69 is not higher than the killer responses of cells pulsed with P3, because the concentration of P69 in both situations was the same.

CD4$^+$ T cell-mediated B cell help was assessed based on the expression of CD69 on FTA B220$^+$ cells [504]. P77 from P3 resulted in a high level of CD69 expression on FTA B cells in the spleen but not in the liver. It is also known that target cells pulsed with P3, which also include FTA B cells, are killed by CD8$^+$ T cells, and this may cause low GMFI of CD69 from target cells due to the low number of cells recovered from vaccinated mice. In addition, from the previous report it was shown that target cells pulsed with NS5B P2 expressed a relatively higher level of GMFI of CD69 [288]. Hence, it was necessary to screen P2 of NS5B to identify peptides of CD4$^+$ T cell targets. Accordingly, target cells pulsed with P2 showed higher GMFI of CD69 and individual peptide analysis revealed that P36 resulted in a significantly higher level of CD69 expression. Peptides in P1 were not screened for the presence of T-cell epitopes because it was previously shown that FTA analysis of P1 loaded target cells were neither significantly lost nor express high level of CD69 [288]. The results obtained, for P2 and P3, in this chapter are also consistent with the previous report [288].

The second aspect of this chapter described the synthesis of an MHC-I tetramer complex that specifically binds to NS5B specific CD8$^+$ T cells. The peptide required for this process is a short amino acid sequence, usually 8-11 amino acids, representing a small portion of the entire antigen [553, 554]. Hence the identification of these immunologically-relevant regions of an antigen is important for the development of methods to monitor T cell responses [555].

The MHC-I-peptide complex was synthesised to track NS5B specific CD8$^+$ T cells and consequently immunodominant peptides recognised by CTL were used as input. P69 and P70 share an 11mer amino acid sequence from which nine amino acids of the sequence were used for the tetramer synthesis. The peptide prediction was performed using the immune epitope data base
(IEDB) analysis resource Consensus tool [556] which combines predictions from ANN aka NetMHC (4.0) [554, 557, 558], stabilised matrix method (SMM) [553] and Comlib [559]. Before the sequence was sent for tetramer synthesis, the peptide manufactured based on this sequence was tested in FTA analysis. Accordingly, the specific loss of FTA target cells loaded with the NS5B\textsubscript{451-459} peptide was comparable to the specific loss of FTA cells loaded with peptide 69 or pool 3. The tetramer was also tested in a pilot experiment for its specificity to NS5B specific CD8\textsuperscript{+} T cells and the results showed insignificant non-specific staining from unvaccinated mice.

4.5 Conclusion
The results in this chapter reported the identification of T cell epitopes of gt3a HCV NS5B using FTA technology. From the analysis, two CTL peptides and two T helper cell peptides were identified. In addition, a nine amino acid sequence NS5B\textsubscript{451-459} peptide was also identified; a MHC-I tetramer, specific to NS5B-specific CD8\textsuperscript{+} T cells, was synthesised based on this peptide and was shown to detect HCV-specific CTL.
Chapter 5: Prime/trap vaccination strategy to induce liver resident memory T cells against HCV

5.1 Introduction
Although circulating memory CD8\(^+\) T cells, which comprise T\(_{CM}\) and T\(_{EM}\) subsets, have protective roles against pathogens, their ability to contain localised infections in tissues and non-lymphoid organs such as the liver is inferior compared to T\(_{RM}\) cells [428, 455]. One of the main reasons for this is the delay in generating a robust inflammatory response required to recruit substantial numbers of T\(_{CM}\) and T\(_{EM}\) cells from the circulation to infected tissues [411]. The presence of robust numbers of CD8\(^+\) T\(_{RM}\) cells which reside in tissues for the lifespan of an individual obviates the requirement of a significant inflammatory response as these cells are well positioned to encounter and eliminate infected cells in tissues soon after infection occurs [560]. The ability of CD8\(^+\) T\(_{RM}\) cells to rapidly control infections in tissues such as the skin, brain, eyes, reproductive tract, the gastrointestinal tract, liver and the lungs has been well established [485-489] making the induction of these cells for T cell based vaccines highly desirable.

Recent seminal studies have highlighted the importance of liver resident T cell responses in controlling and eliminating infections with hepatotropic pathogens [413, 455, 490, 561].

T\(_{RM}\) cells were established in the liver following vaccination of mice with radiation attenuated sporozoites of *Plasmodium berghei* ANKA (PbA). In an attempt to expand these cells in the liver, a prime-trap vaccination strategy was used [455]. The strategy involved priming of malaria specific CD8\(^+\) T cells by using anti-Clec9 antibody-conjugated sporozoite antigen followed by IV vaccination of mice with rAAV encoding the vaccine antigen [455]. T\(_{RM}\) cells induced following this strategy provided sterilising immunity against *P. berghei* challenge [455]. In a human clinical trial, IV delivery of radiation-attenuated *Plasmodium falciparum* sporozoites (PfSPZ) had a cumulative protective efficacy of 55% against repetitive controlled human malaria infections and durable protection against infection was observed for as long as one year [471].
Analysis of immune correlates for protection of the PfSPZ vaccine in non-human primates revealed that protection was strongly correlated with an increased frequency of IFN-γ producing CD8+ T cells in the liver compared with the blood (i.e. in circulation). In fact, the frequency of IFN-γ producing CD8+ T was strikingly higher (100 fold) in the liver than in the blood [471].

These studies suggest that T_{RM} cells in the liver can be protective against hepatotropic pathogen infections and it is likely that these cells will also provide protection against HCV given that HCV, like Plasmodium species, infects hepatocytes. Furthermore, HCV infection appears to be mediated by one or a few variants, referred to as transmitted/founder (T/F) viruses, approximately 100 days before a pool of virus quasi-species emerges [562] making the rapid elimination of HCV extremely desirable. Hence, a vaccine that will induce CMI in the liver is expected to be of paramount importance to prevent the development of persistent virus infection. Thus, the prime/trap strategy as exploited by Fernández-Ruiz et al [455] described above will be adapted using cytolytic DNA (pVAX-NS5B-PRF) to prime HCV-specific T cells and rAAV-NS5B-2A-eGFP, constructed in Chapter 3, to trap the primed cells and encourage these cells to become resident in the liver. The following chapter describes the outcomes of this strategy.

5.2 Aims

1. Assess HCV-specific memory CMI responses following prime/trap vaccination \textit{in vivo}

2. Evaluate whether prime/trap vaccination elicits HCV-specific CD8+ T_{RM} cells

5.3 Results

5.3.1 Vaccination strategy

The results presented in this chapter are derived from a prime/trap vaccination strategy performed as depicted in Figure 5.1. Female BALB/c mice were immunised twice ID with a 50 µg dose of pVAX-NS5B-PRF at fortnightly intervals. Two days after the first pVAX-NS5B-PRF vaccination, the mice were immunised IV with the rAAV-NS5B-2A-eGFP at a dose which results
in ~10% transduction of hepatocytes. Due to the complexity of the experiment in vivo titration of rAAV-NS5B-eGFP in mice was conducted only once.

Figure 5.1: The vaccination schedule to test the ability of a prime/trap strategy to induce T<sub>RM</sub> cells in the liver. Mice were vaccinated ID with two doses of 50 µg DNA (pVAX-NS5B-PRF or pVAX-PRF) fourteen days apart. Two days after the first DNA vaccination, mice received either rAAV-NS5B or PBS via the IV route. Forty-two days (i.e. ‘Day 56’ in the diagram) after the second DNA vaccination the mice were challenged with an FTA. In other experiments with the same vaccination groups and schedules, NS5B-specific CD8<sup>+</sup> were used characterised for their memory phenotype.

This vaccination schedule (Figure 5.1) was selected based on a previous prime/trap vaccination strategy for malaria [455], although the trapping agent was introduced two days after the first vaccination and a DNA boost was introduced 14 days after the first vaccination. The second DNA boost was introduced because it is known that rAAV can persist for up to 3 weeks [382]
which means introducing a second DNA vaccination will cause priming of new CD8 T cells which in-turn became Trm cells due the persistent nature of rAAV. As described in Chapter 3, section 3.2.1.7, 5x10^9 vgc of rAAV-NS5B resulted in transduction of 10.6% mouse hepatocytes and hence this dose was used as a trapping dose. Forty-two days after the final DNA vaccination, some of the mice were challenged with a FTA while others were analysed for the expression of tissue residency markers (CD69, CXCR3 and CD11a) using flow cytometry.

5.3.2 Functional assessment of in vivo cell mediated immune (CMI) response following prime/trap strategy

The FTA to assess CMI responses in vivo were prepared from naïve autologous splenocytes pulsed with either 10 μg/ml of NS5B P1, P2 or P3 peptides or titrated concentrations (0.001, 0.01, 1 or 10 μg/ml) of peptides 36, 77, 69 or the NS5B_{451-459} peptide (Figure 5.2.).

FTA target cells loaded with the different peptide pools and recovered from vaccinated mice showed that the highest in vivo killer responses were directed against P3 of NS5B in all mice vaccinated with pVAX-NS5B-PRF and/or rAAV-NS5B-2A-eGFP (Figure 5.3) which is consistent with results reported previously by our group (13). The robust responses elicited by the trap only group authenticate the use of the rAAV and confirm that NS5B was expressed in vivo. Furthermore, prime/trap and trap only vaccinated mice exhibited the greatest CTL responses against targets loaded with each of the peptide pools (P1-P3) or P69 or NS5B_{451-459} peptide (Figure 5.3). There were no differences in the NS5B-specific CTL responses observed between the prime/trap and trap only vaccinated groups. Both vaccination strategies resulted in > 95% killer response against P3 in the liver and the spleen. When compared to these two vaccination strategies, vaccination with no trap (two 50μg doses of DNA) resulted in significantly lower (P <0.01 both in the liver and spleen) killer responses against P3 (40% in the liver and 60% in the spleen) (Figure 5.3A).
Figure 5.2: Preparation of FTA cells: Splenocytes from syngeneic naïve BALB/c mice were pooled and stained with different concentrations and combinations of CTV and CFSE as shown. Subsequently, the fluorescently bar-coded cells were pulsed with titrated (0, 0.01, 0.1, 1 and 10 µg/ml) concentrations of the indicated peptides or peptide pools. Following peptide loading, all target cells were labelled with 10 µM CPD to delineate the transferred cells from the endogenous population of cells found in FTA challenged mice.

One of the important advantages of the FTA technology is that it allows the analysis of the functional avidity or sensitivity of antigen-specific CD8+ T cells in vivo [503] which was evaluated in this experiment using FTA target cells loaded with titrated concentrations of peptides 69 and NS5B451-459. Accordingly, the prime/trap or trap only vaccinated mice showed higher avidity (sensitivity) killer responses compared to other groups as only mice in these groups elicited responses against targets loaded with the lowest concentration of peptide 69 {i.e. 0.1µg/ml (P = 0.003 relative to no trap group) and 0.01µg/ml (P = 0.017 relative to no trap
The highest avidity response was detected against NS5B_{451-459} peptide and the response showed no decline in targets loaded with 0.01 µg/ml.

Clinical data suggest that a robust correlate of recovery from acute HCV infection is the presence of CD4^{+} T_{H} cells that could mobilise CD8^{+} T cell responses [160]. Effector/memory CD4^{+} T_{H} cells co-stimulate B (B220^{+}) cells presenting cognate antigens resulting in the up-regulation of CD69 on B220^{+} cells [503]. Hence, although measuring T_{H} responses following vaccination is important to assess the effectiveness of a vaccine, the prime/trap strategy described in Fernandez-Ruiz et al. [455] did not assess the contribution of T_{H} cell responses as their strategy was primarily designed to elicit CD8^{+} T_{RM} cells. To address this aspect of the response in the vaccinated mice (Figure 5.1) T_{H} cell responses were measured against the mapped immunodominant peptides 36 and 77 as well as P2 of NS5B.

Mice vaccinated using the prime/trap approach (P=0.002 and P=0.01) and mice vaccinated with the trap only (P=0.009 and P=0.026) elicited the highest magnitude of CD4^{+} T_{H} cell responses to P2 as compared to the no trap group. With respect to the immunodominant T_{H} epitopes, P36 and P77, significant differences in T_{H} cell responses were observed when the FTA cells were loaded with at least 1µg/ml peptide (Figure 5.4B). When FTA cells were loaded with 1µg/ml of P36 or P77, a significantly higher GMFI of CD69 was observed in the spleen of prime/trap (P<0.01) and trap only (P<0.01) vaccinated group as compared to the no trap group. However, in the liver FTA cells loaded with 1 µg/ml of either P36 or P77 did not result in a significant difference in the expression of CD69 between the trap only or the prime/trap vaccinated mice and the no trap vaccinated mice. When FTA cells were loaded with 10 µg/ml of P36 or P77 significant upregulation of CD69 was observed in the trap only vaccinated mice (P = 0.006) as compared to the no trap vaccinated mice. CD69 expression on FTA cells loaded with P3 was not assessed as almost all of these cells were killed by CD8^{+} T cells.
Overall, FTA analysis revealed that prime/trap and trap only vaccination strategies induced superior intrahepatic and systemic CMI than the no trap vaccination strategy.

**Figure 5.3**: FTA analysis of *in vivo* NS5B specific CTL response following vaccination of mice. *In vivo* CTL responses were determined following vaccination of mice using a prime/trap, trap only or no trap (2x DNA only) vaccination regimen. The immune responses were examined 42 days after the final DNA vaccination using FTA technology. A) Bar graphs showing the means (n=7) and SEM of gt3a-specific CTL responses against target cells loaded with 10 µg/ml of NS5B P1, P2 or P3. B) P69 (NS5B<sub>442-459</sub>) and NS5B<sub>451-459</sub> were titrated and loaded on to FTA cells to assess the avidity of induced CTLs. (*P <0.05, **P <0.01).
5.3.3 The prime/trap vaccination strategy induces high frequency of intrahepatic NS5B specific CD8\(^+\) T cells

The frequency of NS5B-specific CD8\(^+\) T cells induced following prime/trap vaccination was analysed using the K\(^d\)NS5B\(_{451-459}\) tetramer and flow cytometry. Double discriminated viable K\(^d\)NS5B\(_{451-459}\) tetramer\(^+\) CD8\(^+\) cells were gated, and analysis of these cells revealed that the majority were found in the liver of mice vaccinated with the prime/trap or the trap only strategy (Figure 5.5A). The percentage and absolute number of NS5B-specific CD8\(^+\) T cells were significantly higher in mice vaccinated with the prime/trap or trap only strategy compared with mice that were vaccinated with the no trap vaccination strategy which mainly induced systemic immunity.

The absolute number of tetramer\(^+\) CD8\(^+\) T cells (includes T\(_{CM}\), T\(_{EM}\) and T\(_{RM}\) cells) in the liver of prime/trap and trap only vaccinated mice was ~8.7 times and ~38 times higher, respectively, than the cells in the no trap vaccinated mice (\(P = 0.009\) and \(P = 0.004\)) (Figure 5.5B). However, a statistically significant difference in the number of systemic (spleen) NS5B specific CD8\(^+\) T cells was not observed between these three vaccination strategies, although there was an increasing trend in the number of cells in those mice that received rAAV-NS5B-2A-eGFP (Figure 5.5B). It is likely that statistical significance would be achieved with greater numbers of vaccinated mice.
Figure 5.4: FTA analysis of *in vivo* NS5B-specific T\textsubscript{H} cell responses. The *in vivo* T\textsubscript{H} response was determined following vaccination of mice using a prime/trap, trap only or no trap vaccination regimen. A) Bar graphs showing the means (n=7) and SEM of GMFI of CD69 expression on FTA B cells pulsed with 10\(\mu\)g/ml of P1 or P2. B) FTA cells were pulsed with titrated concentrations of peptide 69 or 77 and the GMFI of CD69 was determined. (*\textit{P}<0.05, **\textit{P}<0.01).
Figure 5.5: The frequency of NS5B specific CD8⁺ T cells in the liver and spleen. A) Gating strategy to identify tetramer positive CD8⁺ T cells. Lymphocytes were identified by sequential gating SSC-A/FCS-A followed by FSC-W/FSC-H and SSC-W/SSC-H. Dead cells were discriminated using FV620 staining and CD8⁺ T cells were gated on live cells. Tetramer positive cells were gated on CD8⁺ T cells. B) Representative dot plots showing the percentage (in gates) of KdNS5B451-459 tetramer⁺CD8⁺ T cells. C) Percentage and absolute number of NS5B specific CD8⁺ T cells in the liver and spleen of mice vaccinated with the different regimens (*P<0.05, **P<0.01).

In general, in mice vaccinated with the prime/trap or trap only strategy virtually all the CD8⁺ T cells were NS5B specific suggesting that the introduction of rAAV-NS5B was crucial to elicit high numbers of liver-resident NS5B specific CD8⁺ T cells.
5.3.4 The prime/trap vaccination strategy induces high frequency of NS5B-specific T_{RM} cells in the liver

The next logical step to characterise NS5B-specific CD8^{+} T cells was to assess their phenotype for the expression of different markers specific to T_{RM} cells. Liver T_{RM} cells up-regulate CD69 and down-regulate CD62L [455]. Following sequential gating on tetramer^{+} CD8^{+} T cells, the cells were plotted based on CD69 and CD62L expression (Figure 5.6A). As depicted in the representative plot, the majority of the NS5B-specific CD8^{+} T cells in the liver of mice vaccinated with either the prime/trap or trap only strategy were T_{RM} cells (CD69^{+}CD62L^{-}) (Figure 5.6 A). On the other hand, in mice vaccinated with the no trap vaccination regimen, the majority of the NS5B-specific CD8^{+} T cells were T_{EM} cells (CD69^{-}) that were mainly found in the spleen. The results also revealed that all mice vaccinated with prime/trap or trap only strategy showed a high percentage of NS5B-specific T_{RM} cells in the liver (Figure 5.6 B). The absolute number of NS5B specific CD8^{+} T_{RM} cells was significantly higher in the liver of mice vaccinated with the prime/trap ($P = 0.003$) or trap only ($P = 0.003$) strategy as compared to the no trap vaccinated group (Figure 5.6C).

Liver CD8^{+} T_{RM} cells are also known to express high levels of CD11a (CD11a_{hi}) and CXR3 [561]. Consequently, expression of CD11a and CXCR3 was also analysed by assessing the GMFI of these markers (Figure 5.7). Accordingly, statistically significant upregulation of CD11a was observed in NS5B-specific T_{RM} cells induced by the trap only- ($P = 0.004$ as compared to the sham control; $P= 0.003$ as compared to the no trap vaccination) or the prime/trap- vaccination regimen ($P = 0.003$ as compared to sham only control; $P= 0.002$ as compared to the no trap vaccination). There was no significant upregulation of CD11a by T_{RM} cells induced by the no trap vaccination as compared to the sham only control. When compared to the sham only control, CD11a was significantly upregulated by T_{EM} cells induced by- the trap only ($P= 0.006$) or the prime/trap ($P= 0.004$) or the no trap ($P= 0.004$) vaccinations. However, there was no difference
in the upregulation of CD11a by T<sub>CM</sub> cells across the different vaccination groups (Figure 5.7 A). However, analysis of the CXCR3 data showed a high level of background in the sham control. The reason for this was unknown. Flow cytometric analysis showed that in the sham control group, only a small number of events were tetramer<sup>+</sup> CD8<sup>+</sup> in the liver (Figure 5.5B) while even fewer or no events represented NS5B-specific T<sub>EM</sub>, T<sub>RM</sub> and T<sub>CM</sub> cells (Figure 5.6A). Hence the sham control was excluded during statistical comparisons. Nevertheless, CXCR3 was significantly upregulated by T<sub>RM</sub> cells resulting from the trap only vaccination ($P = 0.004$) or prime/trap ($P = 0.01$) vaccination compared to the no trap vaccination. Differences were not observed in the expression of CXCR3 by T<sub>CM</sub> or T<sub>EM</sub> cells across the three vaccination groups (Figure 5.7 B).

Overall, the results suggest that the DNA prime/rAAV trap approach for HCV vaccination elicited CD8<sup>+</sup> T<sub>RM</sub> cells in the liver and significantly improved the magnitude and/or quality of HCV-specific T cell responses <i>in vivo</i> compared to a DNA only vaccination regimen which mainly induced systemic immunity. Equally it was evident from this study that vaccination with rAAV-NS5B alone resulted in a similar level of immune response to the prime/trap vaccination strategy.
Figure 5.6: The frequency of the different subsets of memory CD8⁺ T cells. A) Representative (n = 7/group) dot plots showing expression of CD69 and CD62L on gated KdNS5B451-459 tetramer⁺ CD8⁺ cells from panel A from Figure 5.5. B) The percentage of each memory T cell subset in individual mice. C) The absolute number of each of the CD8⁺ memory T cell subsets across different vaccination strategies. (*P <0.05, **P <0.01).
Figure 5.7: Expression of CD11a and CXCR3 on the different memory T cell subsets isolated from the liver of mice vaccinated with different strategies. GMFI of CD11a (A) and CXCR3 (B) expression on the different intrahepatic memory T cell subsets across the vaccination groups. Representative histogram plots (C) show the expression of CD11a or CXCR3 on NS5B-specific CD8^+^ T_{CM}, T_{RM} and T_{EM} cells. Fluorescent minus one (FMO) plots represent the background staining for CD11a and CXCR3. The GMFI of CD11a (D) or CXCR3 (E) in each vaccine group were compared. Only comparisons between vaccinated groups that had a statistically significant difference are denoted as ‘** p < 0.01 and *** p < 0.001’.
5.3.5 Vaccination with rAAV-NS5B-2A-eGFP results in the persistence of NS5B-specific T\textsubscript{RM} cells in the liver

From the results described above in section 5.2.2 and 5.2.3, it is clear that the no trap (pVAX-NS5B-PRF only) vaccination regimen was less effective than the regimens which included the rAAV-NS5B, particularly with regard to the induction of T\textsubscript{RM} cells in the liver. This does not mean that the DNA vaccination is unable to induce NS5B-specific CD8\textsuperscript{+} T cells as it is possible that NS5B specific CD8\textsuperscript{+} T cells induced from the no trap vaccination strategy were established but not maintained over time. To test this hypothesis, mice were vaccinated as described above (Figure 5.1) but the CD8\textsuperscript{+} T cell characterisation was performed 7 days after the last DNA vaccination. The results were then compared with the results from the 42 days post vaccination.

As depicted in Figure 5.8, significance differences were observed in the number of CD8\textsuperscript{+}tet\textsuperscript{+} cells and T\textsubscript{RM} cells in the liver of mice that did not receive the trapping agent. The results showed that the number of CD8\textsuperscript{+}tet\textsuperscript{+} cells (includes T\textsubscript{RM}, T\textsubscript{CM} and T\textsubscript{EM}-cells) detected 7 days after the final vaccination was significantly ($P = 0.01$) higher than the number detected 42 days after the final vaccination. Similarly, the number of T\textsubscript{RM} cells was significantly higher at day 7 post last vaccination than 42 days post last vaccination. These results suggest the no trap vaccination regimen is capable of inducing antigen (NS5B)- specific CD8\textsuperscript{+} T cells in general and T\textsubscript{RM} cells in particular. However, these cells were not persistently maintained as their number decreased significantly 42 days after the last vaccination. On the other hand, in mice groups that received the trapping agent the number of CD8\textsuperscript{+}tet\textsuperscript{+} cells showed an increasing trend 42 days post final vaccination, although the difference was not significant. This is also true for the number of T\textsubscript{RM} cells in these mice groups. This indicates that the trapping agent contributed to the maintenance of cells for longer period of time, for at least 42 days.
Figure 5.8. A comparison of the frequency of NS5B-specific CD8+ T cells. Mice were vaccinated as described in Figure 5.1 and the results analysed 7 days after the second pNS5B-PRF vaccination were compared with the results from the 42 days post vaccination samples. A. Comparison of CD8+ tetramer+ T cells in the liver and spleen. B. Comparison of TRMs cells in the liver and spleen.

5.4 Discussion

In this chapter the use of a prime/trap vaccination strategy to induce HCV-specific liver TRM cells was described. To achieve this a DNA vaccine encoding the cytolytic protein PRF, in addition to the HCV NS5B protein, was used to prime CD8+ T cells. It is acknowledged that one of the limitations of DNA vaccines is their low immunogenicity and their inability to induce durable T cell immunity [563-565].

Consequently, to improve the immunogenicity of DNA vaccines, a cytolytic protein, PRF, was included in the DNA constructs and used to deliberately induce cell death [287, 288, 566-568].
Cytolytic DNA vaccines were proven to be more immunogenic than canonical DNA vaccines in mice [286, 288] and pigs [287]. These vaccines target cross-presenting dendritic cells and as a result induce increased proliferation of antigen-specific CD8^+ T cells [568].

Although local antigen deposition and antigen encounter is not essential to elicit CD8^+ T_{RM} cells in the skin, the female reproductive tract and the gastrointestinal tract, it is evident that this process leads to the formation of greatest densities of CD8^+ T_{RM} cells especially at sites such as the liver and the vagina [455, 474, 569]. Local antigen encounter/deposition also appears to be crucial to elicit CD8^+ T_{RM} cells in the liver even in humans [455, 471].

DNA vaccines can be delivered using ID and intramuscular routes in a homologous prime/boost manner without plausible anti-vector immunity and thus can be used as a vector to prime high numbers of circulating antigen-specific T cells which can then be recruited to the liver using vectors that efficiently transduce hepatocytes [455]. Considering the poor immunogenicity of DNA vaccines, it was hypothesised that the “prime/trap” vaccination strategy would be a feasible approach in exploiting DNA (as a priming agent) to elicit HCV specific T_{RM} cells in the liver. To test this hypothesis, pVAX-NS5B-PRF was used to prime HCV specific CD8^+ T cells and rAAV-NS5B-2A-eGFP used to trap the primed cells in the liver.

The first section of this chapter showed that the prime/trap and trap only vaccination regimens respectively resulted in superior hepatic and systemic immune responses. As revealed by the FTA challenge experiment, NS5B-specific CTL responses were observed in these groups of mice. Although, this challenge is not an authentic virus challenge model it can be used to assess the effectiveness of the vaccination strategy to induce NS5B-specific CD8^+ T cells. To this end, the observed in vivo CTL responses confirmed that the immune response induced by these two vaccination regimens was robust and induced high avidity T cells. A previous report showed that
mice which were vaccinated against malaria with the prime/trap vaccination strategy were protected from *P. berghei* infection [455].

Further analysis of the location of the NS5B-specific CD8$^+$ T cells was conducted using tetramer staining of cells isolated from the liver and spleen. Accordingly, a significantly higher proportion of NS5B specific CD8$^+$ T cells was localised in the liver of mice vaccinated with the prime/trap or trap only vaccination strategy. In contrast, the number of cells in the spleen was not significantly different between the groups. This indicates that a single dose of rAAV-NS5B-2A-eGFP was sufficient to attract high numbers of NS5B specific CD8$^+$ T cells into the liver. Similarly, a prime/pull vaccination strategy resulted in a high frequency of antigen specific CD8$^+$ T cells in the vagina while the frequency of systemic antigen-specific CD8$^+$ T cells in the spleen was not significantly changed [495].

Phenotypically, T$_{RM}$ cells are CD69$^+$CD62L$^-$ cells [455, 461]. The results from this study also showed that NS5B-specific CD8$^+$ T$_{RM}$ cells isolated from the liver of mice vaccinated with prime/trap or trap only vaccinations were CD62L$^-$CD69$^+$. Other markers of T$_{RM}$ cells like CXCR3 and CD11a [433, 434] were also highly expressed by these cells. On the other hand, the phenotype of cells in mice vaccinated with the no trap strategy showed a higher frequency of T$_{EM}$ cells and this was more pronounced in the spleen than the liver, confirming the results of a previous study [455].

The introduction of the trapping agent in the current vaccination strategy contributed to the high frequency of NS5B specific CD8$^+$ T cells in general and T$_{RM}$ cells in particular in the liver of mice consistent with the fact that local antigen deposition is crucial to elicit CD8$^+$ T$_{RM}$ cells in the liver [455, 471].

Studies in mice and humans suggest that IV immunisation is the most efficient route to facilitate local antigen encounter in the liver [382, 471, 570]. Imaging of live mice injected ID or IV with
*P. berghei* sporozoites suggested that the sporozoites were reproducibly detected in the liver only after IV injection, while the parasite load in the liver was 30-fold higher in the IV injected mice [570]. The same study also showed that IV compared to ID injection of irradiated sporozoites elicited a significantly higher proportion of CD62L⁻ memory CD8⁺ T cells and increased protection against live sporozoite challenge [570]. ID, SC and intramuscular immunisation of humans with radiation-attenuated *P. falciparum* sporozoites (PfSPZ) also appeared to elicit suboptimal immunity and protection against malaria compared to IV vaccination [471, 571]. Analysis of vaccinated non-human primates suggest that the increased efficacy of IV PfSPZ vaccination in humans correlated strongly with the ability of this vaccine to elicit long lived liver-resident CD8⁺ T cells [471, 571]. Furthermore, transduced hepatocytes have the capacity to present AAV-encoded antigens to CD8⁺ T cells following IV vaccination with AAV [382] and analysis of liver biopsies from end stage hepatitis C patients suggest that HCV infection elicits CD11hi memory CD8⁺ T cells [572]. Collectively these findings are consistent with our findings which showed that ID vaccination with pVAX-NS5B-PRF was less efficient in eliciting intrahepatic T cell immunity and CD8⁺ T_{RM} cells compared to IV rAAV-NS5B vaccination.

5.5 Conclusion

In this chapter the contribution of a prime/trap or trap only vaccination strategy to induce a robust CMI against HCV is described. As a result of the rAAV-NS5B-2A-eGFP introduction to the liver, a high frequency of NS5B specific T_{RM} cells was observed in the liver. The efficacy of a DNA vaccine to develop a T cell-based vaccine against HCV will likely rely on its ability to elicit robust immune response in the liver. Considering the poor immunogenicity of DNA vaccines this seems difficult to achieve unless a strategy is designed to overcome this situation. In this regard the prime/trap vaccination strategy described in this chapter could be a potential strategy to elicit HCV specific T_{RM} cells in the liver that can act as a frontline defence against the virus.
6 Chapter 6: General Discussion

Vaccines are of great public health importance and the previous success of the smallpox and poliovirus vaccines suggest that vaccination is a cost-effective means to eliminate HCV infections worldwide [243].

A recent mathematical modelling study used empirical data to simulate HCV transmission amongst people who inject drugs and showed that an effective vaccine could reduce transmission rates by >90% during the first months of exposure in vaccinated individuals compared to naïve individuals [573]. An effective vaccine is considered essential to meet the WHO’s Sustainable Development Goal of eliminating 90% of global hepatitis C incidence and viral hepatitis as a major public health threat by 2030 [574]. However, there is no licensed vaccine for HCV. The inability to reliably induce rare immune correlates of protection following vaccination and the lack of convenient animal models permissive to infection are significant issues that have contributed to this fact [252, 575]. After two decades of unsuccessful preclinical studies and Phase I HCV vaccine trials, the current lead candidate is an NIH-sponsored Phase IIb, placebo-controlled trial in high risk people who inject drugs utilising a prime-boost regimen of gt1 NS antigens delivered via ChAd and then modified vaccinia virus Ankara. However, this vaccine only induces systemic T cell immunity to gt1b antigens [284]. Assuming that the AAV alone is equally effective as the ChAd/MVA vaccine (although the efficacy of neither has been determined), then the AAV vaccine will be more cost effective.

The efficacy of any HCV vaccine based on CMI will be dependent on the speed of T cell migration to the liver and consequently a vaccine which elicits and maintains intrahepatic HCV-specific CD8+ T RM cells and CD4+ T cell immunity has an intrinsic advantage as the immune cells are resident in the liver. Robust liver-resident CD8+ T cell response elicited following vaccination were shown to be crucial for protection against controlled human malaria infection in humans [471] and P.berghei infection in mice [455].
Consequently, in this thesis, pre-clinical evaluation of a vaccination strategy to elicit intrahepatic HCV-specific CD8\(^+\) T\(_{RM}\) cells is described for the first time. The strategy involved ID vaccination of mice with pVAX-NS5B-PRF followed by IV vaccination with rAAV-NS5B-2A-eGFP to improve CD8\(^+\) killer T cell and CD4\(^+\) helper T cell responses in vaccinated mice. A previous study exploiting AAV as a vector to elicit intrahepatic immunity used AAV encoding minitopes and TCR transgenic T cells to evaluate resident CD8\(^+\) T cell response in adoptive transfer setting [455]. The study described in this thesis exploited vaccine vectors encoding codon-optimised gt3a full length NS5B protein and evaluated T cell immunity in a polyclonal repertoire in wild-type mice. Furthermore, the study described the use of a novel FTA assay to map immunodominant CD4\(^+\) T helper cell and CD8\(^+\) T cell epitopes of gt3a NS5B in vivo unlike previous studies which used in vitro peptide stimulation assays to map T cell epitopes. Poor survival of liver resident T cells, particularly CD8\(^+\) T\(_{RM}\) cells, following in vitro peptide stimulation highlights the importance of exploiting the FTA assay. The in vivo epitope mapping analysis led to the development of a functional MHC-I tetramer and the identification of NS5B-specific CD4\(^+\) T helper cell immunodominant peptides. The prime/trap vaccination strategy improved CD8\(^+\) T killer cell and T\(_H\) cell responses in vaccinated mice as determined using tetramer and FTA challenge analysis. This was observed both in systemic (spleen) and intrahepatic T cell responses. However, the avidity and magnitude of systemic and intrahepatic T cell responses elicited were similar following trap only vaccination compared to the prime/trap vaccination.

6.1 AAV vector as a vaccine
Viral vectors are capable of inducing outstanding antibody responses and also elicit CTL that are crucial for control of intracellular pathogens [576]. The immune responses generated from viral vector vaccines are directed against the encoded heterologous antigen as well as the vector itself [577]. Thus, concerns associated with pre-existing immunity to-and the strong immunogenicity of- viral vectors are main barriers to the use of viral vectors in vaccine formulations [577]. Despite
these limitations, Adenovirus and pox virus based viral vector vaccines are the most widely investigated recombinant vaccines [506, 577-579].

The use of rAAV vectors has been explored since the first report by Manning et al. that documented the ability of intramuscular rAAV to induce a strong humoral and cellular responses against HSV type 1 glycoprotein B [580]. The overall absence of toxicity in rAAV-mediated gene therapy made AAV vectors attractive vectors in vaccine development [506]. rAAV vectors were evaluated in the development of vaccines for HIV [581, 582] and were able to reach to Phase I clinical trial [583]. Intranasal and intramuscular administration of rAAV encoding HIV env, tat and rev resulted in the production of high levels of HIV-specific antibodies [508]. Later oral administration of rAAV encoding HIV env was shown to induce local and systemic CMI and partially protected mice against rectal challenge with recombinant vaccinia virus encoding the HIV env [581]. A more advanced study in macaques investigated the efficacy of rAAV vectors encoding simian immunodeficiency (SIV) proteins [582]. This study demonstrated that a single vaccination induced robust T cell and antibody responses which were protective against low dose IV SIV challenge [582]. One phase I clinical trial revealed that rAAV-2 encoding HIV-gag, -protease and part of the reverse transcriptase was safe although it showed modest immunogenicity as only 16% of vaccinated individuals developed Gag-specific T cells [583]. Other reports also showed the progress made in the use of rAAV vectors in vaccine development against HPV [525, 584, 585] and influenza virus [511, 586], at least in animal models. More recently a rAAV vaccine was used to trap T cells in the liver of mice following priming of these cells in the spleen using a radiation attenuated sporozoite vaccine [455]. In this study radiation attenuated sporozoite vaccination was used to prime malaria-specific CD8+ T cells in the spleen and rAAV encoding the vaccine antigen was used to trap primed cells in the liver. The immune response elicited by this vaccine was able to induce protective immunity against sporozoite challenge [455]. These studies suggested that rAAV vector vaccines can be used in vaccines development against HCV.
In this thesis, rAAV-NS5B-2A-eGFP was used to trap T cells in the liver, after these cells were primed by the cytolytic DNA vaccine, pVAX-NS5B-PRF. To ensure efficacy, it was necessary to confirm that gene expression was localized to the liver as antigen deposition leads to a higher number of TRM cells [455, 474, 569]. This was achieved by two mechanisms viz the use of the liver specific hAAT promoter [519] and the use of AAV capsid, AAV 8, that is known to transduce mouse hepatocytes [530, 531, 587]. It is estimated that 7-20% of hepatocytes will be HCV infected during persistent HCV infection [588]. It has also been shown that a CTL response was maintained when less than 25% of hepatocytes were antigen positive following vaccination [382]. Using eGFP expression to quantitate antigen-expressing hepatocytes, 5 x 10^9 vgc of rAAV-NS5B-2A-eGFP was able to transduce 10.6% of hepatocytes and this dose was used in the vaccination strategy. Previously it was reported that 5x10^10 or 1 x 10^11 vgc of rAAV encoding eGFP and/or mOVA transduced 100% of hepatocytes in vivo [381, 382, 528]. When the dose was lowered to 5x10^8 vgc small proportion of hepatocytes were transduced although the percentage of antigen-expressing hepatocytes was not indicated [382]. The level of eGFP expression described in this thesis is lower than the previous reports and this may be due to the larger size of the gene inserted in the current study. It is known that rAAV encoding larger inserts has lower level of transduction [317, 385, 386].

The main limitation of AAV 8-mediated gene transfer for either gene therapy or vaccination is the requirement of a relatively higher vector dose due to its low capacity to transduce human hepatocytes [587, 589]. Hence, apart from using a tissue-specific promoter to localize antigen expression, selection of an effective AAV serotype that can transduce the target tissue is necessary. Capsid engineering has been proposed as a strategy to improve the clinical efficacy of AAV-based vectors by making the viral capsid unrecognized by the immune system [371]. Paulk et al., reported rAAV with a bioengineered capsid that showed improved levels of transduction in mice xenografted with human liver [590]. According to this report, the new variants (NP40 and
NP59) significantly reduced the seroreactivity profile as compared to AAV 8 [590]. Although ensuring scalable production of rAAV based on synthetic peptides and understanding the prevalence of anti-capsid antibodies in the general population are important steps in the use of synthetic peptides, the achievements suggest that AAV-based vaccines represent a potential strategy for the development of vaccines for HCV.

6.2 T cell epitope mapping

Vaccine development requires measurement and understanding of the adaptive immune response induced following vaccination. The analysis of the CD8+ T cell response is of particular interest in vaccine development for viral infections [535, 591]. T cell functional analysis using methods like the 51Cr-release assay [592] has several drawbacks including its reliance on cell lines that can take up 51Cr, high spontaneous release of 51Cr from cells over time, its semiquantitative nature, inter-assay variability, the requirement to restimulate CTLs prior to the assay, its biohazardous nature and its inability to directly assess cell killing in vivo [535]. On the other hand, ELISpot assays are only surrogate markers as they do not directly measure cytotoxic activity [591]. Flow cytometry-based techniques to detect target cell death by CTL activity are emerging methods of CTL immune responses [593-596]. Continuous improvement of the flow cytometry methods led to the development of the FTA technology, a technique that is used to measure CMI in vivo [535, 552]. FTA technology can be considered the most useful, not only because it is performed in vivo, but also because it has the capacity to assess killing of multiple targets presenting different concentrations and different types of peptide epitopes, which is required to assess functional avidity of CD8+ T cells [503]. The assay can also provide information on CD4+ T cell responses [503, 504].

In this thesis the FTA technology was used to identify immunodominant peptides of the vaccine antigen, HCV NS5B, from an array of 90 peptides following vaccination of mice with pVAX-
NS5B-PRF. From the analysis two CTL immunodominant peptides and two T\textsubscript{H} immunodominant peptides were identified (Chapter 4). CTL immunodominant peptides were found in P3 and T\textsubscript{H} cell immunodominant peptides were identified from P2 and P3. Whether these peptides will be responsible for immune response in humans will be an area of further exploration. According to the human clinical trial reports based on the human Ad/ChAd and ChAd/MVA vaccination, that encoded NS3-NS4A-NS4B-NS5A-NS5B from HCV gt 1B, it was described that NS3 was the immunodominant peptide pool from the NS proteins encoded in the vaccine [283, 284, 597, 598]. Peptide tracking was done in smaller peptide pools, rather than individual peptides, and this also confirmed that the immunodominant region was located in NS3 [283]. Based on these observations it is difficult to make direct comparison to the vaccination described in this thesis as the genotype tested here is HCV gt3a where we identified NS5B as an immunodominant peptide that contained CD8\textsuperscript{+} T cell epitopes. Moreover, there were no reports that analysed individual peptides of HCV NS proteins for immunodominance in human clinical trial. Hence, it will not be conclusive to state that NS5B will be “immunogenic” or “not immunogenic” in humans.

Quantitative analysis and phenotyping of antigen-specific T cells is advanced by the development of fluorochrome-conjugated peptide-MHC multimers [599-601]. Hence, to further characterize and quantitate CD8\textsuperscript{+} T cells, antigen-specific CD8\textsuperscript{+} T cells were first discriminated using peptide MHC-I tetramer staining. In order to synthesize the tetramer, the CTL immunodominant peptides identified previously were used as an input. Tetramer design for MHC-II restricted T cells is complex due to the weaker affinity TCR on CD4\textsuperscript{+} T cells [602-604]. As a result, a MHC-II tetramer was not used in the study described in Chapter 5.

6.3 Prime/trap vaccination strategy to induce robust CMI in the liver
DNA vaccines are simple to design, cheap to manufacture and, due to their stability, they do not require cold chain for storage and transportation [285, 605-608]. DNA vaccines are non-viable which avoids the chance of reversion to a disease-causing agent [609]. Furthermore, DNA
vaccines do not induce vector-mediated immune responses which is an advantage if multiple doses of an antigen are desirable [610]. These qualities made DNA-based vaccines attractive vaccine delivery candidates.

DNA vaccines encoding HPV E6 and E7 resulted in histological regression and/or eliminated persistent HPV infection and HPV-related cervical lesions [611, 612]. More recently, a DNA vaccine was developed that induced protective NAb to Zika virus (ZIKV) in mice [613] and rhesus macaques [565] which have also led to the development of safe and immunogenic ZIKV DNA vaccines in humans [614, 615].

However, DNA vaccines are unable to induce long-term immune responses following a single or a few vaccinations [565]. To overcome their poor immunogenicity, DNA vaccines have been modified to allow improved uptake of the vaccine antigen by DC by conjugation with anti-Clec9A and anti-DEC2015 antibodies to target DC surface receptors [455, 616, 617]. However, this strategy required the use of additional adjuvants [566]. As a result, more recently DNA vaccines that encode the cytolytic protein, PRF, were developed [287, 566-568, 618]. These vaccines were developed to achieve cell death-induced innate immune activation to enhance antigen uptake by DC and hence improve the immunogenicity of vaccines [619, 620]. Following necrotic cell death intracellular factors will be released and act as DAMPs that are capable of signaling DC [566, 621]. The addition of PRF as a genetically encoded adjuvant within a DNA vaccine has been shown to increase immune response to HCV-NS3 [287], HCV-NS3, 4 and 5 [286] and HIV gag [567].

The recent emergence of DNA vaccines in eliciting protective immunity in humans and higher animal models warrants further examination as to how DNA vaccines can be harnessed in vaccination regimens to target HCV. However, DNA vaccines are not optimized to elicit localised
immunity in the liver suggesting a requirement for further refinements of DNA-based vaccination regimens in order to elicit lifelong protection against HCV.

The concept of priming circulating antigen-specific T cells and trapping activated cells in different organs like the liver [455] and vaginal epithelial cells [474, 495] has been tested and induced protective immune responses. Given the poor transfection efficiency and immunogenicity of DNA when delivered into the liver, this strategy appears to be a more feasible approach in exploiting DNA (as a priming agent) to elicit HCV-specific T$_{RM}$ cells in the liver.

The work described in Chapter 5 of this thesis aimed to develop a strategy to induce localised CMI against HCV in the liver. The CMI response after vaccination using the prime/trap vaccination strategy (Chapter 5, section 5.3.1) was assessed in mice. The strategy involved vaccination of mice with two 50µg doses of pVAX-NS5B-PRF 14 days apart. Two days after the first vaccination the mice received rAAV-NS5B-2A-eGFP IV and the immune response was measured 42 days after the final DNA vaccination (Chapter 5 section 5.3.2), to ensure that memory as opposed to acute responses were measured.

Ideally, the assessment of T cell responses is best performed in vivo with no manipulation of T cells to avoid changes in functional parameters [552]. To achieve this FTA was used to assess CMI responses resulted from prime/trap vaccination strategy. This technology is particularly important for the in vivo analysis of T$_{RM}$ cells as these cells survive poorly in functionality assays performed in vitro [433, 455].

FTA analysis showed that the prime/trap vaccination strategy induced significantly higher CTL and T$_{H}$ cell immune responses. The induced CTL were also of high avidity as they were able to recognise FTA cells loaded with 0.01µg/ml peptide. Previously it has been shown that, in mice vaccinated with a cocktail of DNA vaccines encoding gt1b NS5B and gt3a NS5B, the CTL killer response was higher against FTA cells loaded with P3 of gt3a NS5B [288]. Although the avidity
of CTL cells induced following this vaccination not reported, the overall killer response against FTA cells loaded with P3 was similar to that reported in this thesis. In the prime/trap vaccination strategy described by Fernandez-Ruiz et al., although the trapping agent, rAAV-NVY, alone was able to induce high numbers of T_{RM} cells in the liver, protection from sporozoite challenge was achieved only when T_{RM} cells were generated after the prime/trap vaccination rather than the rAAV-NVY alone [455]. In contrast, the result described in this thesis showed that vaccination with rAAV-NS5B-2A-eGFP alone was able to induce significantly higher number of T_{RM} cells and these cells showed higher percentage killer response towards peptide-loaded FTA cells as compared to the no trap vaccination. The use of a rAAV-based vaccine on its own against HCV represents a potential area of investigation for future studies.

Phenotypic characterisation of HCV antigen-specific CD8^+ T cells, described in section 5.3.4, revealed that cells isolated from the liver of mice that received the trapping agent expressed high levels of CD69, CXCR3 and CD11a which are described as residency markers [433, 434, 440]. The frequency of T_{RM} cells was also higher in mice that received the rAAV-NS5B-2A-eGFP compared with mice that did not receive the trapping agent. Although the necessity of local antigen deposition to elicit CD8^+ T_{RM} cells is controversial, it is believed that antigen deposition led to a high frequency of these cells in the liver and vagina [455, 471, 474, 569]. In the results described in this thesis, antigen deposition from rAAV-NS5B-2A-eGFP contributed to the high frequency and persistence of T_{RM} cells. Comparison of the frequency of T_{RM} cells 7 days and 42 days after the second DNA vaccination showed longer maintenance of T_{RM} cells in mice that received rAAV-NS5B-2A-eGFP suggesting its role in the persistence of these cells.

Overall, rDNA and rAAV based vaccination strategies that can be exploited to elicit systemic and intrahepatic HCV-specific T cell immunity which have ramifications for the design of effective vaccines against hepatotropic pathogens such as malaria, HBV and HCV were evaluated. This
study also highlights the importance of exploiting hepatotropic vaccine vectors to elicit robust and durable intrahepatic cytotoxic CD8$^+$ T cell immunity and Th cell responses against HCV in vivo. Specifically, it appears that AAV alone will represent the most efficient approach.

6.4 Future studies
The results described in this thesis support further testing of the prime/trap vaccination strategy both in small and large animal models. The results from future studies will be crucial for further evaluation of the vaccination strategy in human clinical trials.

6.4.1 Simplifying the prime/trap vaccination strategy
The vaccination strategy described in this thesis involved a three-stage vaccination approach. Such multiple encounters coupled with the IV route used to deliver rAAV-NS5B-2A-eGFP may be an obstacle to translate this vaccination approach to the clinic. However, IV vaccine delivery was used in a recent malaria vaccine clinical trials and was reported as painless by the volunteers [471, 622]. The results described in this thesis also suggest that a single IV rAAV-NS5B injection is more immunogenic than two dose ID DNA vaccination. Although it has been described that the IV route is more effective route, as compared to ID or SC routes, for antigen deposition in the liver [382, 471, 570] the procedure is still complex as compared to other vaccines delivery routes. Hence, comparing the IV route ID or SC routes may be a way forward to develop the simplest possible strategy for rAAV delivery.

6.4.2 Trm depletion studies
Although the FTA challenge studies showed strong CMI responses and a high frequency of Trm cells following prime/trap vaccination strategy, the immune response was not evaluated in the absence of Trm cells. A previous study used anti-CXCR3 to deplete Trm cells in the liver [455], however the results in this thesis showed that this marker was also expressed by other subsets of memory T cells. Hence it was difficult to perform depletion studies. Hence, future studies should consider the identification of specific biomarkers for the establishment Trm cells in the liver. On
the other hand, it was reported that $T_{RM}$ cells attract $T_{EM}$ cells in different organs [623]. However, the relationship between $T_{RM}$ cells and $T_{EM}$ cells was not investigated and the role of $T_{EM}$ cells during FTA challenge was not assessed. Thus, it will be important for future studies to demonstrate these immunological interactions. In the transgenic mouse model study, it was reported that $T_{RM}$ cells in the liver associated with liver sinusoids [455]. However, it is not determined if this is also true for wild type mice in the context of HCV. Hence it will be important to investigate the location of these cells in the liver.

**6.4.3 Generating a universal HCV vaccine**

The induction of broad CMI that can protect against heterologous HCV strains to cover a range of HCV genotypes is important for the development of a universal vaccine against HCV [161, 288]. Equally, an effective HCV should ideally induce NAb, because passive transfer of these antibodies showed protection from heterologous virus in mice and chimpanzees [624-626]. Therefore, a vaccine covering viral heterogeneity able to induce a strong NAb response and a vigorous multi-specific broad CMI may be necessary to overcome viral immune escape.

The proof of concept study described in this thesis needs further exploration for the development of a universal HCV vaccine based on the prime/trap vaccination. One of the main hurdles to achieve is the low cargo capacity of AAV [384] that will require the use of multiple viral vectors to accommodate multiple HCV genes from multiple genotypes [627, 628]. Although insufficient to allow packaging of full-length genomes, minimising the size of regulatory elements may provide additional space to accommodate transgenes. To this end promoters like CMV can be shortened from ~600bp to 173bp [629] while the 300bp SV40 poly A sequence can be replaced by a 32bp-long polyadenylation of soluble neurophilin 1 without significant reduction in gene expression [630]. Moreover, the rAAV used in this study encodes eGFP to allow easy analysis of transduction efficacy. Complete removal of eGFP or changing eGFP to a smaller size, like iLOV [631], should be considered. Together these amendments may allow the insertion of another HCV
gene or if this is not possible this will still provide an advantage as it will minimize the genome size and improve the transduction efficacy.

6.4.4 HCV challenge model to test efficacy of prime/trap vaccination strategy
The protective efficacy of a vaccination strategy is best evaluated using authentic virus challenge. Chimpanzees, considered the optimal animal model to test HCV vaccine efficacy, are endangered and their use is subject to stringent ethical considerations limiting the use of these animals in preclinical studies of candidate vaccines [5, 188, 192]. Hence, developing alternative small and large animal models is necessary to test HCV vaccines before human clinical trials. As far as large animal models are considered, pigs are very similar to humans in terms of anatomy, genetics and physiology with > 80% similarity in the immune system [632]. Pigs were used to test immunogenicity of a candidate HCV vaccine [287]. Hence these animals can be alternative large animal model to test the immunogenicity of the prime/trap vaccination strategy.

The immunocompetent transgenic mouse models that support HCV infection would be helpful to evaluate the protective efficacy of prime/trap vaccination strategy [207, 633]. The GB Virus B (GBV-B) has been shown to infect marmosets and results in HCV-like disease [634]. A HCV/GBV-B chimera, containing HCV structural genes, developed by Li et al., was able to induce persistent infection in marmosets which suggests the potential of these animal models to test vaccine efficacy [635]. Another HCV/GBV-B chimera containing the HCV NS2-4A genes was also capable of infecting marmosets resulting in persistent viremia with typical viral hepatitis including histopathological changes in the liver tissue [636]. Both chimeras would be useful to test the protective efficacy of the prime/trap or trap only strategy in a non-human primate, prior to future human clinical trials.

6.4.5 Conclusion
The use of DNA in the development of T cell-based HCV vaccine will likely rely on its ability to be exploited in a regimen that can elicit robust immunity in the liver. In this regard, with further
studies, the prime/trap or trap only vaccination strategy described in this thesis should be considered for the development of vaccination strategies to elicit immune responses, localised in the liver, against HCV.
7 Appendices

7.1 Appendix I: Table of Chemicals and reagents

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<tr>
<th>Chemical/Reagent</th>
<th>Manufacturer</th>
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<tbody>
<tr>
<td>2-mercaptoethanol</td>
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</tr>
<tr>
<td>30% acrylamide/Bis solution</td>
<td>BioRad</td>
</tr>
<tr>
<td>Absolute ethanol</td>
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<tr>
<td>Acetic acid</td>
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<tr>
<td>Agar</td>
<td>AMRESCO</td>
</tr>
<tr>
<td>Agarose</td>
<td>AMRESCO</td>
</tr>
<tr>
<td>Albumin (bovine)</td>
<td>Sigma-Aldrich</td>
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<tr>
<td>Ammonium chloride</td>
<td>Sigma-Aldrich</td>
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<tr>
<td>Ammonium persulfate</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Boric acid</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Chloroform</td>
<td>Sigma-Aldrich</td>
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<td>DAPI (4’6-Diamindino-2-Phenylindole, Dihydrochloride)</td>
<td>ThermoFisher Scientific</td>
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<td>DMEM</td>
<td>ThermoFisher Scientific</td>
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<td>DMSO</td>
<td>Sigma-Aldrich</td>
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<tr>
<td>EDTA</td>
<td>Sigma-Aldrich</td>
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<td>Foetal calf serum (0.1um filtered)</td>
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<tr>
<td>GenElute</td>
<td>Life Technologies</td>
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<tr>
<td>Glucose</td>
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<td>Chemical</td>
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<td>Glycine</td>
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<td>HEPES</td>
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<td>Isopropanol</td>
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<td>Kanamycin</td>
<td>Sigma-Aldrich</td>
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<td>Lipofectamine® LTX with Plus™ Reagent</td>
<td>Thermofisher Scientific</td>
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<td>Magnesium chloride</td>
<td>VWR International</td>
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<td>VWR International</td>
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<td>Sigma-Aldrich</td>
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<td>Sigma-Aldrich</td>
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<td>Non-fat dried milk</td>
<td>Black and Gold</td>
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<td>optiMEM</td>
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<td>Penicillin/streptomycin</td>
<td>Life Technologies (Gibco)</td>
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<td>Phosphate-buffered saline</td>
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<tr>
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<td>Sigma-Aldrich</td>
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<tr>
<td>Polyethyleneimine (PEI)</td>
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<tr>
<td>Potassium bicarbonate</td>
<td>Sigma-Aldrich</td>
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<td>Thermofisher Scientific</td>
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<td>Thermofisher Scientific</td>
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<td>RPMI 1640</td>
<td>Life Technologies</td>
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<td>Ingredient</td>
<td>Supplier</td>
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<td>------------------------------------</td>
<td>-------------------------</td>
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<td>Sodium Chloride</td>
<td>ChemSupply</td>
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<td>Sodium dihydrogen phosphate</td>
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<tr>
<td>Sodium Dodecyl Sulphate</td>
<td>Sigma-Aldrich</td>
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<tr>
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<td>Sigma-Aldrich</td>
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<tr>
<td>Sodium pyruvate</td>
<td>Life Technologies</td>
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<td>TEMED</td>
<td>Sigma-Aldrich</td>
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<tr>
<td>Triton-X</td>
<td>BioRad</td>
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<td>Trizma-base</td>
<td>Sigma-Aldrich</td>
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<tr>
<td>Trizol</td>
<td>Life Technologies</td>
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<tr>
<td>Trizol</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>Trypsin</td>
<td>Life Technologies (Gibco)</td>
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<tr>
<td>Tryptone</td>
<td>Becton Dickinson and Company</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>Becton, Dickinson and Company</td>
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### 7.2 Appendix II: Table of equipment

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Manufacturer</th>
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</thead>
<tbody>
<tr>
<td>Platform shaker</td>
<td>Heidolph Instruments</td>
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<tr>
<td>Heat block</td>
<td>BioRad Digital dry bath</td>
</tr>
<tr>
<td>Bacterial shaker</td>
<td>Ratek Orbital mixer incubator</td>
</tr>
<tr>
<td>Bacterial spectrophotometer</td>
<td>BioRad Smartspec 3000</td>
</tr>
<tr>
<td>Small table-top centrifuge</td>
<td>Sigma 1-15PK</td>
</tr>
<tr>
<td>Large table-top centrifuge</td>
<td>Eppendorf centrifuge 5810R</td>
</tr>
<tr>
<td>Large centrifuge</td>
<td>Beckman T2-21M/E centrifuge</td>
</tr>
<tr>
<td>Bacterial incubator</td>
<td>Labmaster Anax Division</td>
</tr>
<tr>
<td>Cell culture incubator</td>
<td>SANYO CO₂ incubator</td>
</tr>
<tr>
<td>Real-time PCR cycler</td>
<td>Corbett Rotor-Gene 3000</td>
</tr>
<tr>
<td>Western blot imager</td>
<td>FujiFilm LAS-4000</td>
</tr>
<tr>
<td>Flow cytometer</td>
<td>BD Biosciences FACScanto</td>
</tr>
<tr>
<td>Fluorescence microscope</td>
<td>Zeiss AxioLab inverted Microscope</td>
</tr>
<tr>
<td>Agarose gel imager</td>
<td>BioRad Gel DocXR system</td>
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</tbody>
</table>
### 7.3 Appendix III: Table of Kits

<table>
<thead>
<tr>
<th>Kit</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pierce™ BCA protein Assay Kit</td>
<td>ThermoFisher Scientific</td>
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<tr>
<td>PreLink® Quick gel Extraction and PCR purification Combo kit</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>PureLink® Quick plasmid Miniprep kit</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>KAPA high fidelity PCR kit</td>
<td>KAPA biosystems</td>
</tr>
<tr>
<td>KAPA Taq PCR kit</td>
<td>KAPA biosystems</td>
</tr>
<tr>
<td>AAVpro® Titration kit for Real time PCR</td>
<td>Takara Bio Inc</td>
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</table>
### 7.4 Appendix IV: Bacterial Growth media

<table>
<thead>
<tr>
<th>Media/solution</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luria-Broth (LB)</td>
<td>10 g Tryptone, 10 g NaCl, 5 g yeast extract, adjust to 1 L MQ H₂O</td>
</tr>
<tr>
<td>LB agar</td>
<td>10 g Tryptone, 10 g NaCl, 5 g yeast extract, 15 g agar, adjust to 1 L MQ H₂O</td>
</tr>
<tr>
<td>TfbI solution</td>
<td>30 mM KAc, 100 mM KCl, 10 mM CaCl₂, 50 mM MnCl₂, 15% glycerol, water. Adjust the pH to 5.8 with acetic acid</td>
</tr>
<tr>
<td>TfbII solution</td>
<td>10 mM MOPS, 10 mM KCl, 75 mM CaCl₂, 15% glycerol, water. Adjust the pH to 6.5 with KOH</td>
</tr>
<tr>
<td>Psi-a media</td>
<td>6 g yeast extract, 24 g tryptone, 6 g MgSO₄ adjust the pH to 7.6 with KOH (final volume 1.2 L)</td>
</tr>
<tr>
<td>Psi-b agar</td>
<td>Add 2.8 g of Agar to 200 ml of Psi-a media</td>
</tr>
<tr>
<td>SOC Media</td>
<td>20 g tryptone, 5 g yeast extract, 0.58 g NaCl, 0.19 g KCl, 2.03 g MgCl₂, 2.46 g MgSO₄, 3.6 g glucose (Final volume 1 L using MQ H₂O)</td>
</tr>
</tbody>
</table>
### 7.5 Appendix V: Media for mammalian cell culture growth and media for lymphocyte isolation

<table>
<thead>
<tr>
<th>Medium</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplemented DMEM</td>
<td>500 ml DMEM, 50 ml FCS, 5 ml pen/strep</td>
</tr>
<tr>
<td>R-10</td>
<td>RPMI-1640, 10% FCS, 25 mM HEPES, 1 mM Sodium Pyruvate, 1x 2ME, 1% Pen/Strep</td>
</tr>
<tr>
<td>R-5</td>
<td>RPMI-1640, 5% FCS, 25 mM HEPES, 1 mM Sodium Pyruvate, 1x 2ME, 1% Pen/Strep</td>
</tr>
<tr>
<td>Supplemented HBSS</td>
<td>HBSS, 3% FCS, 1% Pen/Strep</td>
</tr>
<tr>
<td>Isotonic percoll</td>
<td>90% percoll, 10% supplemented HBSS</td>
</tr>
</tbody>
</table>
### 7.6 Appendix VI: List of labelling dyes and antibodies

<table>
<thead>
<tr>
<th>Labelling dye or antibody</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTV</td>
<td>Eugene</td>
</tr>
<tr>
<td>CFSE</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>CDP</td>
<td>eBioscience</td>
</tr>
<tr>
<td>Fixable viability dye (FV620)</td>
<td>BD Bioscience</td>
</tr>
<tr>
<td>Anti-CD44 (FITC)</td>
<td>Biolegenden</td>
</tr>
<tr>
<td>Anti-CD11a (BV510)</td>
<td>DB Bioscience</td>
</tr>
<tr>
<td>Anti-CXCR3(BV421)</td>
<td>BD Bioscience</td>
</tr>
<tr>
<td>Anti-CD69 (PE-Cy7)</td>
<td>BD Bioscience</td>
</tr>
<tr>
<td>Anti-CD62L (PE)</td>
<td>BD Bioscience</td>
</tr>
<tr>
<td>Anti-CD8 (AF780)</td>
<td>eBioscience</td>
</tr>
<tr>
<td>NS5B tetramer-APC</td>
<td>Biomolecular Research Facility, the Jhon Curtin School of Medical Research, Canberra</td>
</tr>
<tr>
<td>Alex-fluor 555 goat anti-human IgG</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>
# 7.7 Appendix VII: SDS-PAGE buffers

<table>
<thead>
<tr>
<th>Solution</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Running gel buffer</td>
<td>40 mM Tris, 185 mM Glycine, 0.1% SDS</td>
</tr>
<tr>
<td>Resolving gel buffer</td>
<td>2 M Tris HCl pH8.8, 10% SDS, 30% Acrylamide</td>
</tr>
<tr>
<td>Stacking gel buffer</td>
<td>2 M Tris HCl pH6.8, 10% SDS, 30% Acrylamide</td>
</tr>
<tr>
<td>5 x SDS loading buffer</td>
<td>100 mM Tris HCl pH6.8, 2% SDS, 2 ml 10% glycerol, 5% β-mercaptoethanol, 1 µg/ml bromophenol blue</td>
</tr>
<tr>
<td>Running buffer</td>
<td>6.25 ml Tris pH8, 6.25 ml Tris pH8.8, 10 ml 10% SDS, 14.4 g glycine, to 1000 ml MQ H₂O</td>
</tr>
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</table>
### 7.8 Appendix VIII: Primers used for cloning, sequencing and RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer name</th>
<th>Primer sequence (5’ to 3’) (Lower case= restriction enzyme, Upper case only= overhang, italic = kozak sequence, Bold= start codon, Bold &amp; Italic= stop codon, underlined= region of homology to the gene of interest)</th>
<th>Restriction site</th>
</tr>
</thead>
<tbody>
<tr>
<td>eGFP</td>
<td>2A-eGFP forward</td>
<td>AAgaattcAAACTTCGACCTGCTGAAGCTGGCC GCGACGTGGAGAGCAACCCCGCCCAGCAGGCTGGAGGCGACGTGGAGAGCAACCCCGCCCAGCAGGCTGGAGGCGACGTGGAGAGCAACCCCGCCCAGCAGGCTGGAG</td>
<td>EcoRI</td>
</tr>
<tr>
<td></td>
<td>eGFP reverse</td>
<td>TTTagcttt <strong>TTACTTGTACAGCTCGTCCATGCGAG</strong>, GGCGACGTGGAGAGCAACCCCGCCCAGCAGGCTGGAGGCGACGTGGAGAGCAACCCCGCCCAGCAGGCTGGAG</td>
<td>HindIII</td>
</tr>
<tr>
<td>HCV gt3a</td>
<td>NS5B forward</td>
<td>AAAggggecgc <strong>GCCCCCATGCTATGCTACTCTTGGAC</strong>, GGCGACGTGGAGAGCAACCCCGCCCAGCAGGCTGGAGGCGACGTGGAGAGCAACCCCGCCCAGCAGGCTGGAG</td>
<td>NotI</td>
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<tr>
<td></td>
<td>Ns5B reverse</td>
<td>TTgaattc<strong>TCACCGAGCTGGCAGGAAGATG</strong>, GGCGACGTGGAGAGCAACCCCGCCCAGCAGGCTGGAGGCGACGTGGAGAGCAACCCCGCCCAGCAGGCTGGAG</td>
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<tr>
<td>hAAT</td>
<td>hAAT sequencing primer</td>
<td>GATCCCAGCCAGTGGACTTAGCC</td>
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<tr>
<td>HCV gt3a</td>
<td>RT-PCR forward 1</td>
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<tr>
<td>RT-PCR</td>
<td>reverse 1</td>
<td>CACGCT GCGGAT CTG GTTGATGGCTC</td>
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<td>-----------------</td>
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<tr>
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<td>GAGCCG CTTGGGAGACTGCCAG</td>
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<td>reverse 2</td>
<td>GTCTCCAAGCCCGCAGTGAGGAC</td>
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<td>forward 3</td>
<td>GCCTGCTAGAGAATTCACTTCAGGACCTG</td>
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<td></td>
<td>reverse 3</td>
<td>GGGCAGCTTGCGGTGGTGCA</td>
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<td>CATCCTGGGGCACAAGCTGGAGTAC</td>
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<td></td>
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8 References


