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# Polyfunctional CD8$^+$ T cells are associated with the vaccination-induced control of a novel recombinant influenza virus expressing an HCV epitope

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ABSTRACT

In hepatitis C virus (HCV) infection, CD8$^+$ T cell responses have been shown to be important in viral clearance. Examining the efficacy of CD8$^+$ T cell vaccines against HCV has been limited by the lack of an HCV infectious model in mice and the differences between MHC restriction in humans and mice. Using HLA-A2 transgenic HHD mice, we demonstrate that intranasally delivered Pam2Cys-based lipopeptides containing HLA-A2-restricted HCV epitopes can induce polyfunctional CD8$^+$ T cell responses in several organs including the liver. To examine the activity of these responses in an infectious context, we developed a recombinant influenza virus that expresses the NS5B$_{2594–2602}$ epitope from non-structural protein 5B of hepatitis C virus (PR8-HCV$_{NS5B}$). We showed that mice inoculated with a lipopeptide containing the NS5B epitope had reduced viral loads following challenge with the PR8-HCV$_{NS5B}$ virus. This reduction was associated with the induction of NS5B$_{2594–2602}$-specific IFN-γ and TNF-α co-producing CD8$^+$ T cells. The T cell receptor usage in the NS5B$_{2594–2602}$ response was found to exhibit a Vβ8.1/8.2 bias that was characterized by a narrow repertoire and a common CDR3β motif. This work has identified CD8$^+$ T cell functions induced by lipopeptides that are associated with viral control and demonstrate the potential of lipopeptide-based vaccines as candidates for treatment of HCV infection.



## 1. Introduction

Hepatitis C virus (HCV) infection affects up to 3% of the world's population (http://www.who.int) and the high incidence of chronic disease is associated with an increased risk of developing hepatitis, cirrhosis, and liver cancer (Heintges and Wands, 1997; Shepard et al., 2005). Conventional HCV treatment involves a costly regime of pegylated interferon-α and ribavirin combination therapy and success in treatment is highly dependent on the HCV genotype and patient viremic levels (Fried et al., 2008; Manns et al., 2001). There is therefore an urgent need to develop preventive and/or therapeutic vaccination strategies to improve current anti-HCV treatment.

Approximately 26% of HCV infections are spontaneously cleared (Micallef et al., 2006) with clearance being associated with a vigorous and broad CD8$^+$ T cell response (Gruner et al., 2000; Lechner et al., 2000; Missale et al., 1996; Thimme et al., 2001). In particular, epitopes derived from the NS5B or NS3 internal proteins of HCV have been associated with spontaneous resolution of infection or linked to treatment-associated recovery (Lechner et al., 2000; Smyk-Pearson et al., 2006; Vertuani et al., 2002). In contrast, the HCV-specific CD8$^+$ T cell responses in chronically infected patients are weaker, narrower in specificity and functionally impaired in terms of cellular proliferation, TNF-α and IFN-γ production (Gruener et al., 2001; Nisii et al., 2006; Wedemeyer et al., 2002) and cytotoxicity (Penna et al., 2007). The liver, the primary site of HCV replication, has been reported to be an immunosuppressive IL-10 rich environment that is poorly-conducive to the priming of effector CD8$^+$ T cells (Crispe et al., 2000; Holz et al., 2010), potentially contributing to the limited and sub-optimal activity of HCV-specific responses.

Although there is strong evidence that supports the role of CD8$^+$ T cells in the control of HCV infection, the inability of mice to be infected with HCV has limited laboratory-based assessment of effective treatment and vaccination strategies. Attempts to address this problem have been made with surrogate challenge models using, for example, recombinant vaccinia virus (Arribillaga et al., 2002; Murata et al., 2003) and gamma herpes viruses (El-Gogo et al., 2008) that express HCV proteins or epitopes. These models have been useful in identifying targets of CD8$^+$ T cell responses such as NS3 proteins, related to viral protection (El-Gogo et al.,

*Abbreviations:* HCV, hepatitis C virus; ICS, intracellular cytokine staining; i.n., intranasal; IAV, influenza A virus; LP, lipopeptide.

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2008). More recently, it has been shown that expression of human CD81 and occuldin genes in mice facilitates HCV infection of mouse hepatocytes *in vivo* (Dorner et al., 2011). This transgenic mouse therefore provides a method for examining immune responses to HCV infection in an immunocompetent animal model. However, because the animal is not transgenic for human class I molecules, it does not allow a study of those class I HLA-restricted $CD8^+$ T cell epitope responses that are observed during human HCV infection.

The advent of reverse genetics (Hoffmann et al., 2000) has recently enabled the engineering of select $CD8^+$ T cell epitopes into relatively innocuous viral vectors such as influenza A viruses (Sexton et al., 2009). The advantage of influenza-based vectors is that these can be constructed in mouse-adapted viruses and hence pose minimal health risks to the user. Such recombinant influenza viruses provide surrogate challenge models that facilitate the quantitative and functional characterization of $CD8^+$ T cell effector populations during active infection, when a direct challenge model is unavailable. In this study we make use of reverse genetics to construct a recombinant influenza virus, PR8-$HCV_{NS5B}$, in which the epitope $NS5B_{2594–2602}$ from HCV is engineered into the neuraminidase protein of influenza virus PR8.

The focus of this study was to examine the ability of Pam2Cys-lipopeptides to induce multiple $CD8^+$ T cell epitopes to HCV and to evaluate *in vivo* immunity utilizing a recombinant influenza virus PR8-$HCV_{NS5B}$. Pam2Cys-based lipopeptides are synthetic, epitope-based constructs that contain the Toll-like receptor 2 (TLR-2) agonist S-[2,3-bis(palmitoyloxy)propyl]cysteine (Pam2Cys) and have been demonstrated to be protective in a number of different challenge models (Deliyannis et al., 2006; Jackson et al., 2004; Lau et al., 2006; Zeng et al., 2002). These lipopeptides include a single $CD8^+$ T cell epitope and a T helper epitope which is required for generating long lasting $CD8^+$ T cell memory responses (Deliyannis et al., 2002).

Although the liver is a poor site for priming hepatic $CD8^+$ T cells, intranasal infection with influenza A virus is highly efficient at inducing cytotoxic and functional $CD8^+$ T cell responses in this organ (Keating et al., 2007; Polakos et al., 2007). A previous study has shown that intranasal and subcutaneous infection with adenovirus also induces functional $CD8^+$ T cell responses in the liver, but infection by the intravenous route induces dysfunctional hepatic antigen-specific $CD8^+$ T cells (Lukens et al., 2009). These results suggest that the route of inoculation can dictate the efficiency of $CD8^+$ T cell priming (Lukens et al., 2009). We therefore hypothesized that vaccination by the intranasal route could induce functional $CD8^+$ T cells in the liver and have direct relevance for HCV-based treatment. In this study, we examined the immunogenicity of lipopeptides containing HCV-derived epitopes following intranasal vaccination. We demonstrate that multi epitope lipopeptide inoculation is able to induce polyfunctional $CD8^+$ T cell responses at multiple organs including the liver in immunized HHD mice. We have also shown that lipopeptide-induced $CD8^+$ T cell responses to the $NS5B_{2594–2602}$ epitope reduce the viral burden and accelerate viral clearance following challenge with a recombinant influenza virus containing the $NS5B_{2594–2602}$ epitope. In doing so we have identified a role for $CD8^+$ T cell polyfunctionality, but not T cell repertoire diversity, in mediating viral clearance.

## 2. Methods

### 2.1. Peptide synthesis

Lipopeptide and peptide syntheses were performed by conventional solid phase Fmoc chemistry as described previously (Zeng et al., 2002). The lipopeptides were assembled as a branched structure (Fig. 1A) and consisted of the IA restricted ovalbumin-derived $CD4^+$ T helper cell epitope (OT2) with amino acid sequence ISQAVHAAHAEINEAGR (Robertson et al., 2000), linked to a $CD8^+$ T cell epitope ($T_{CD8}$) through an intervening lysine residue. The T helper epitope was selected based on restriction compatibility to the genetic background of the HHD transgenic mice and the previously reported dominance in C57BL/6 mice (Robertson et al.) The HCV-specific lipopeptides contained the following HLA-A2 restricted HCV-derived $CD8^+$ T cell epitopes; ALYDVVTKL ($NS5B_{2594–2602}$), CINGVCWTV ($NS3_{1073–1081}$), KLVALGINAV ($NS3_{1406–1415}$) and YLVAYQATV ($NS3_{1585–1593}$). The control lipopeptides, Core-LP and $HSV_{gB}$-LP contained the $T_{CD8}$ epitopes HCV $Core_{132–140}$ (sequence DLMGYIPLV) or epitope $gB_{498–505}$ (sequence SSIEFARL) derived from herpes simplex virus-1 (HSV) (Wallace et al., 1999), respectively. Peptides representing the $CD8^+$ T cell epitopes alone were also synthesized for use in *in vitro* re-stimulation assays.

Peptides and lipopeptides were purified by reversed phase high performance liquid chromatography (RP-HPLC) using a semi-preparative Vydac C4 column (10 mm × 250 mm) column installed in a Waters HPLC system. Chromatographic analysis was conducted at a flow rate of 1 ml/min using 0.1% TFA in double distilled water and 0.1% TFA in acetonitrile. The authenticity of products was determined using analytical HPLC where the products eluted in a single major peak indicating a purity of >95% and product was verified using mass spectroscopy using an Agilent 1100 Series ion trap mass spectrometer (Agilent Technologies, Santa Rosa, CA).

### 2.2. Animals

Female HLA-A2 transgenic HLA-$A2K^b$ (Vitiello et al., 1991) and HHD (Firat et al., 1999; Pascolo et al., 1997) mice aged between 6–12 weeks were used. HHD mice were kindly provided by Dr. François Lemonnier from the Institut Pasteur (Paris, France) and breeding pairs were supplied by Professor Andreas Suhrbier (Queensland Institute of Medical Research, Australia). Mice were bred and maintained in the Animal House Facility, Department of Microbiology, The University of Melbourne.

### 2.3. Ethics statement

All animal experimentation was conducted following the Australian National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Scientific Purposes guidelines for housing and care of laboratory animals and performed in accordance with institutional regulations after pertinent review and approval (approval ID: 04178, 06061, 0911091) by the University of Melbourne Animal Ethics Experimentation Committee in Melbourne.

### 2.4. Generation of the recombinant PR8-$HCV_{NS5B}$ influenza virus

The recombinant influenza virus, PR8-$HCV_{NS5B}$, was generated using an eight-plasmid reverse genetics system (Hoffmann et al., 2000) and the $NS5B_{2594–2602}$ epitope was inserted into the influenza virus neuraminidase (NA). NA-specific fragments were amplified using universal primers and internal primers were designed for the $NS5B_{2594–2602}$ epitope containing the terminal Bsa1 sites:

5′-TAATGGTCTCTGATGTTGTTACTAAACTTAACCATACTGGAA-TATGC-3′ and
5′-TAATGGTCTCACATCATAAAGAGCTTGACTTCCAGTTTGAATT-GAATG-3′.

PCR products were digested with BsaI (10,000 U/ml, Biolabs, Ipswich, MA) and ligated into the pHW2000 cloning vector. The

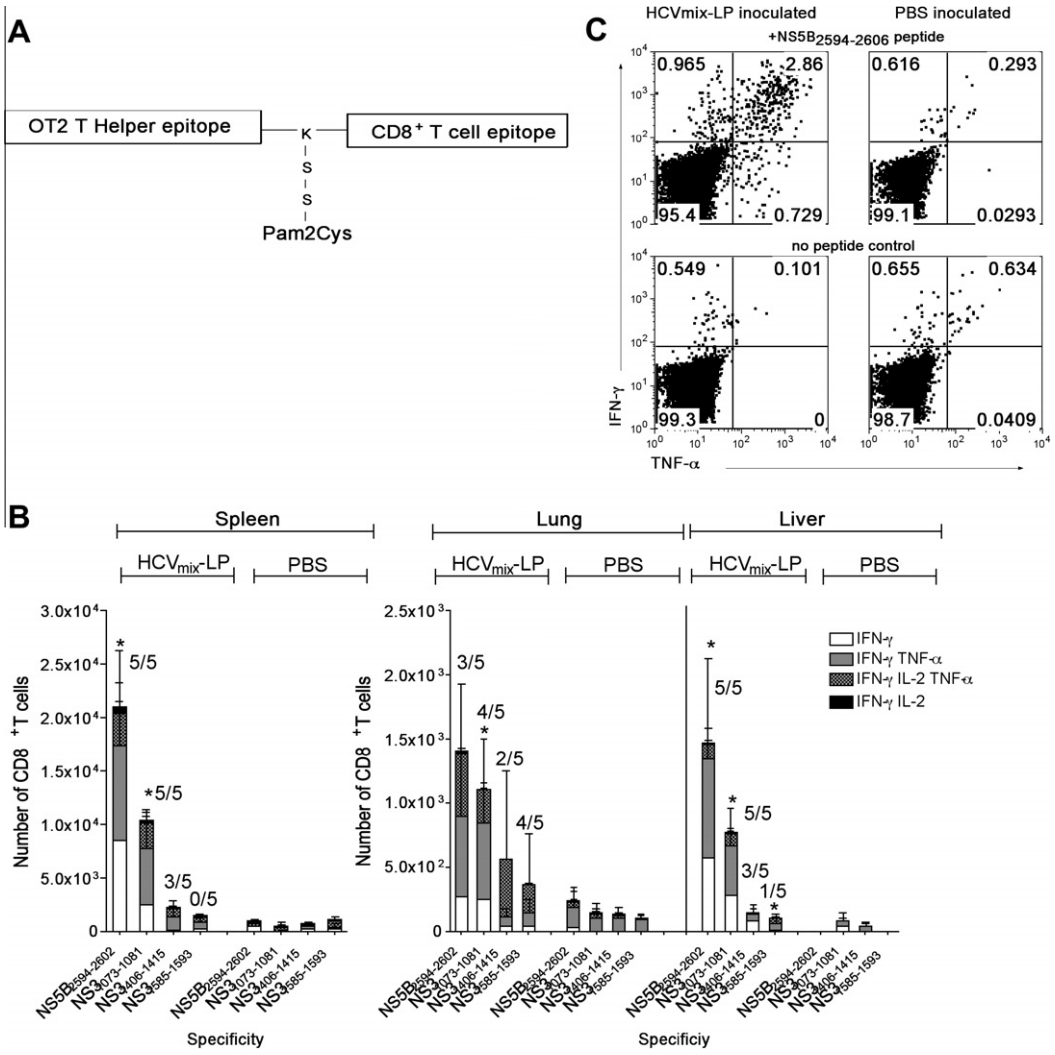


**Fig. 1.** Pam2Cys-based lipopeptides induce multiple specificities of polyfunctional HCV-peptide-specific CD8$^+$ T cells. (A) Pam2Cys-based lipopeptides comprise a peptide backbone containing the T helper epitope OT2 and an HLA-A2-restricted CD8$^+$ T cell epitope linked by an intervening lysine residue. The Pam2Cys moiety is conjugated to the peptide backbone by two serine residues. (B) HHD mice were inoculated (i.n.) with $HCV_{mix}$-LP, a mixture of four HCV lipopeptides ($n = 5$) or PBS ($n = 3$). Ten days later the CD8$^+$ T cell responses to each of the included HCV-derived epitopes was detected from the spleen, lung and liver cells by *ex vivo* ICS assay. Bars represent the mean response to each epitope in each group and the segments comprise the different cytokine producing populations. The $*$ symbol represents significant differences in the epitope-specific CD8$^+$ T cell response compared to the PBS group where $P < 0.05$ (Mann–Whitney *t*-test). The values ($n$/5) indicate the number of mice with positive responses to the epitope, defined as a value greater than value + 2xSD of the PBS response. (C) Representative FACS plots for the IFN-γ and TNF-α cytokine profile of the NS5B-specific CD8$^+$ T cells in a $HCV_{mix}$-LP inoculated or PBS inoculated mouse. Numbers indicate the percentage of cells in the respective quadrant.

recombinant virus was rescued after transfection of eight plasmids encoding influenza nucleic acid segments into mixed cultures of 293T and MDCK cells (Hoffmann et al., 2000). The recombinant influenza A viruses were grown in the allantoic fluid of embryonated eggs and viral titers determined by plaque assay. A haemagglutinin (HA) assay was used to confirm the generation of functional virus and the presence of the $NS5B_{2594–2602}$ insert was confirmed by extraction of viral RNA by RT-PCR and subsequent RNA sequencing.

### 2.5. Inoculation and viral challenge

For intranasal (i.n.) administration of lipopeptide or virus, mice were anaesthetized by inhalation of Penthrane™ or isoflurane. Lipopeptide was dissolved in 50 μl of saline, and HHD mice were inoculated i.n. with either a single 35 nmol dose of an individual lipopeptide, or a mixture of four lipopeptides ($HCV_{mix}$-LP) at 25 nmol each. HLA-A2K$^b$ mice were inoculated i.n. with two 50 nmol doses of lipopeptide, 2 weeks apart. PR8-$HCV_{NS5B}$ recombinant virus was administered i.n. at 50 or 200 plaque forming units (pfu) in 50 μl of PBS.

### 2.6. Preparation of single-cell suspensions

Spleens and lungs were processed into single cell suspensions as described in Tan et al. (2011). Liver cells were obtained by perfusing livers through the hepatic portal vein with 10 ml of PBS to remove circulating lymphocytes. The liver was washed in FACS wash (PBS containing 1% FCS and 0.1 M EDTA) and then resuspended in 25 ml of isotonic, 33.75%, Percoll (Amersham Biosciences/Pharmacia). The Percoll mixture was centrifuged at 693*g* for 12 min at room temperature. The cell pellet containing liver-derived lymphocytes was treated with ATC and washed in RP10 RP10 (RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum (FCS) (CSL, Parkville, Australia), 7.5 mM HEPES, 2 mM L-glutamine, 76 μM 2-mercaptoethanol, 150 U/ml penicillin, 150 μg/ml streptomycin and 150 μM non-essential amino acids

(Gibco)). Live cells were counted on a haemocytometer using trypan blue exclusion.

### 2.7. Intracellular cytokine staining assay (ICS)

ICS was performed on 2–3 $\times$ $10^6$ spleen, lung or liver cells as described in Tan et al. (2011). Cells were fixed and permeabilized using the BD Cytofix/Cytoperm kit (BD Biosciences Pharmingen) according to manufacturer's instructions, and cells were stained with either FITC-labeled anti-IFN-$\gamma$ APC/PeCy7-labeled anti-TNF-$\alpha$ and APC-labeled IL-2 (BioLegend, San Diego, CA) or surface stained with FITC-labeled anti-CD43, PerCP-Cy5.5-labeled anti-CD27 (BD Pharmingen) and PE-Cy-7 labeled anti-CD8 followed by intracellular staining with PE-labeled anti-TNF-$\alpha$ and APC-labeled-anti-IFN-$\gamma$ (BioLegend, San Diego, CA) antibodies for 30 min at 4 °C. Samples were analyzed using a Becton Dickinson FACSCalibur or FACS Canto flow cytometer and the data analyzed using FlowJo software (Treestar, Inc., Ashland, OR).

### 2.8. T cell restimulation and IFN-$\gamma$ ELISPOT assay

In HLA-A2K$^b$ mice, splenocytes were harvested 7 days following the second dose of immunogen and subsequently re-stimulation *in vitro* as described by Chua et al. (2008). Briefly, re-stimulated cells were incubated for 18 h in the presence of irradiated autologous splenocytes and 10 μg/ml of peptide ($NS5B_{2594–2602}$).

### 2.9. Determination of viral titers

Titers of infectious virus in the lungs of infected mice were determined by plaque assay on monolayers of Madin-Darby kidney (MDCK cells) as previously described (Tannock et al., 1984).

### 2.10. $NS5B_{2594–2602}$ tetramer staining and TCR Vβ chain usage

The HLA-A2 monomer complexed to $NS5B_{2594–2602}$ peptide (Monomer Facility, Department of Microbiology and Immunology, The University of Melbourne) was used to generate tetramer with streptavidin-conjugated allophycocyanin (APC) (Molecular Probes). Lymphocytes were stained with 1/100 dilution of tetramer (1 μg/ml) in FACS wash for 60 min at room temperature, followed by two washes in sort buffer (0.1% BSA in PBS). Cells were labeled with Per-CP Cy5.5 conjugated anti-mouse CD8α antibody (BD Pharmingen) and a single chain anti-TCR Vβ antibody (Vβ 2; 3; 4; 5.1/5.2; 6; 7; 8.1/8.2; 9; 10, 11, 12, 13 or 17, BD Pharmingen) for 30 min at 4 °C. Samples were washed twice and HLA-A2 expression determined by flow cytometry.

### 2.11. Isolation and phenotyping of single $CD8^+$ T cells

Lymphocytes were isolated from the spleens of lipopeptide inoculation-PR8-$HCV_{NS5B}$-infected mice 10 days following infection and stained with APC-conjugated $NS5B_{2594–2602}$ tetramer, PE-anti-CD8α antibody (BD Pharmingen) and either FITC anti-Vβ4 or FITC-anti-Vβ8.1/8.2 antibodies (BD Pharminigen). The NS5B-tetramer$^+$ $CD8^+$ Vβ4$^+$/Vβ8.1/8.2$^+$ cells were isolated with a MoFlo sorter (Cytomation, Fort Collins, CO) fitted with a Cyclone single-cell deposition unit. Cells were sorted directly into a 96-well PCR plate (Eppendorf) and single cell RT-PCR and sequencing performed as described previously (Kedzierska et al., 2004).

### 2.12. Statistical analyses

Statistical analysis was carried out using GraphPad Prism software. Unpaired, two-tailed students's *t*-test was used to compare the results obtained between two groups, and one-way ANOVA with post hoc Tukey's multiple comparison test was used to compare results between >2 group. Differences were considered significant when $P$ values were <0.05 with a 95% confidence level.

## 3. Results

### 3.1. Lipopeptides induce multispecific and polyfunctional HCV-peptide-specific $CD8^+$ T cell responses in HLA-A2 transgenic mice

Pam2Cys-based lipopeptides are epitope-based constructs which incorporate a T helper and $CD8^+$ T cell epitope in a branched configuration with the TLR2 agonist Pam2Cys (Fig. 1A). Lipopeptide and the corresponding $CD8^+$ T cell epitopes were synthesized using solid phase F-moc chemistry and the products were purified using by reversed phase high performance liquid chromatography. Analytical HPLC confirmed the purity (>95%) of the products, which eluted in a single major peak and mass spectrometry was used to verify the authenticity of the products.

A multi-specific $CD8^+$ T cell response is considered to be essential for optimal control of HCV. To induce multiple $CD8^+$ T cell responses, we constructed a panel of Pam2Cys-based lipopeptides each containing a single HLA-A2-restricted $CD8^+$ T cell epitope derived from HCV. The epitopes were selected based on their reported association with spontaneous resolution of infection, or with viral clearance following treatment (Lechner et al., 2000; Smyk-Pearson et al., 2006; Vertuani et al., 2002) and included $NS5B_{2594–2602}$ from the NS5B protein and $NS3_{1073–1081}$, $NS3_{1406–1415}$ or $NS3_{1585–1593}$ from the NS3 non-structural protein of HCV. Responses to these lipopeptides were assessed in HHD transgenic mice. These transgenic mice express a chimeric form of HLA-A2.1 and have previously been shown to elicit a similar repertoire of HCV-specific responses as those seen in the infected human population (Himoudi et al., 2002), highlighting the usefulness of such transgenic animals in the evaluation of HCV-epitope specific responses. HHD mice were inoculated intranasally (i.n.) with a mixture of the four lipopeptides referred to as the $HCV_{mix}$-LP mixture and $CD8^+$ T cell responses were examined 10 days later. At day 10 following inoculation, we identified a response to all epitopes (Fig. 1B and S1). However the strongest responses were directed at $NS5B_{2594–2602}$ and $NS3_{1073–1081}$, which could be detected in the spleens, lung and livers of all inoculated mice. Lipopeptide-induced $CD8^+$ T cells predominantly produced IFN-$\gamma$ alone or IFN-$\gamma$ and TNF-$\alpha$. A 6 day *in vitro* restimulation of the spleen cells confirmed the immunodominance of the $NS5B_{2594–2602}$ and $NS3_{1073–1081}$ responses which increased to 53 ± 8% and 53 ± 7% of the $CD8^+$ T cell population, respectively, whilst $NS3_{1406–1415}$ and $NS3_{1585–1593}$ populations expanded to 4 ± 4% and 4 ± 3% of the $CD8^+$ T cell population, respectively (data not shown). This demonstrates that delivering several lipopeptides simultaneously can induce multiple specificities of $CD8^+$ T cells, albeit at different magnitudes. This type of immunodominance hierarchy, where the magnitude of responses are not equally induced has been observed previously (Tan et al., 2011), and is likely to be an inherent trait of multi-epitope vaccination.

### 3.2. A recombinant PR8-$HCV_{NS5B}$ influenza virus induces $CD8^+$ T cells recognizing an HCV epitope

A current predicament for the development and evaluation of an anti-HCV $CD8^+$ T cell-based vaccine is the lack of an HCV that is adapted for use in an immune competent mouse model. We therefore constructed a novel recombinant influenza virus expressing the HCV $NS5B_{2594–2602}$ epitope on an infectious PR8 influenza A virus background (PR8-$HCV_{NS5B}$) using reverse genetics (Hoffmann et al., 2000) with the objective of using this as a tool to measure

*in vivo* lipopeptide-induced HCV immunity. We have already shown that the lipopeptide-induced $NS5B_{2594–2602}$-specific responses are immunodominant and unlike $NS3_{1073–1081}$, this epitope has not been shown to cross-react with influenza A virus (Urbani et al., 2005; Wedemeyer et al., 2001), making it suitable to incorporate into the recombinant influenza virus.

To verify the infectivity of the recombinant PR8-$HCV_{NS5B}$ virus and the correct expression of the NS5B insert, HHD mice were infected i.n. with 50 pfu of virus and on day 5 and 10 following infection the presence of $CD8^+$ T cell responses to the $NS5B_{2594–2602}$ epitope were examined. In mice infected with PR8-$HCV_{NS5B}$, we were able to detect $NS5B_{2594–2602}$-specific $CD8^+$ T cells *ex vivo* in spleens and lungs at 10 days following infection (Fig. 2A and B) but not at day 5 post-infection, a time point of infection which is considered too early to detect $CD8^+$ T cell responses (Lau et al., 2006).

### 3.3. Characterizing the lipopeptide-induced $NS5B_{2594–2602}$-specific $CD8^+$ T cell response

The following studies were performed to characterize the functional profile and activity of lipopeptide-primed $CD8^+$ T cells during PR8-$HCV_{NS5B}$ virus infection. This would reveal whether lipopeptide-primed $CD8^+$ T cells could recognize the $NS5B_{2594–2602}$ epitope presented in an infectious context such as PR8-$HCV_{NS5B}$ challenge.

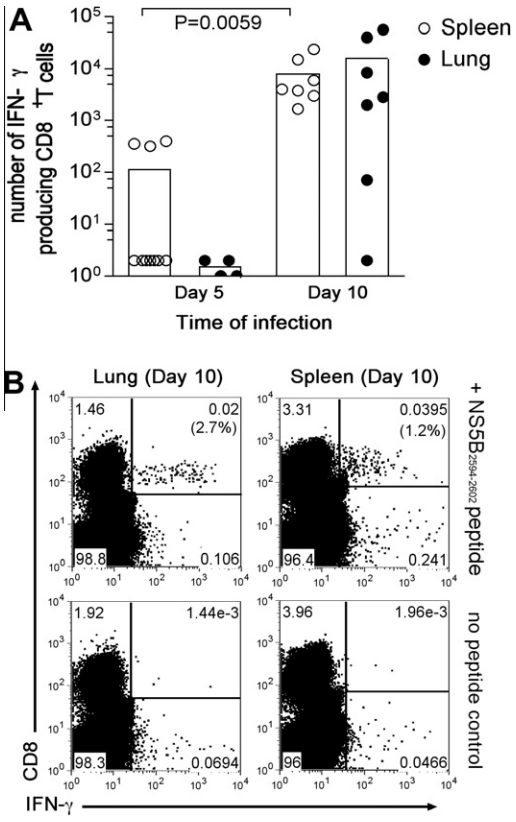


**Fig. 2.** Infection with PR8-$HCV_{NS5B}$ virus induces $NS5B_{2594–2602}$-specific $CD8^+$ T cells. HHD mice were challenged (i.n.) with 50 pfu of PR8-$HCV_{NS5B}$ and at day 5 and 10 following infection $NS5B_{2594–2602}$ specific $CD8^+$ T cells were detected using an IFN-γ detecting ICS assay. The total number of spleen or lung $NS5B_{2594–2602}$-specific $CD8^+$ T cells on days 5 and 10 post-challenge are shown in (A). *P* values indicate significant differences between the vaccination groups (one-way ANOVA with post hoc Tukey's multiple comparison test). Symbols represent the response obtained in individual animals and the bars signify the mean response of the group. (B) Representative FACS plots of the IFN-γ positive $CD8^+$ T cells on day 10 of infection. Numbers indicate the percentage of cells in the respective quadrant and numbers in brackets indicate the % of the IFN-$\gamma^+$ portion of $CD8^+$ T cells. The chart (A) combines results obtained from two separate experiments.

Mice were inoculated with a single lipopeptide containing the $NS5B_{2594–2602}$ epitope (NS5B-LP), or a control lipopeptide (Core-LP) that contains an HCV-epitope not found in the PR8-$HCV_{NS5B}$ virus. Lipopeptide-inoculated mice were challenged 60 days later with 200 pfu of virus.

In the group that received NS5B-LP, a significant number of IFN-$\gamma^+$ and IFN-$\gamma^+$ TNF-$\alpha^+$ $NS5B_{2594–2602}$-specific $CD8^+$ T cells were present in the spleen on day 6 after challenge, while only negligible responses were detected in the control PBS and Core-LP groups (Fig. 3A and B and S2). We were also able to identify IFN-γ and IFN-γ, TNF-α co-producing $NS5B_{2594–2602}$-specific $CD8^+$ T cells in the liver and bronchoalveolar lavage fluid (BAL) in the group receiving NS5B-LP but not in animals receiving PBS or the Core-LP. Based on the observations that at day 6 after infection, we could only detect $NS5B_{2594–2602}$-specific $CD8^+$ T cell responses in mice inoculated with NS5B-LP but not the control groups, is consistent with this being an infection-induced recall response. This indicates that the $NS5B_{2594–2602}$ epitope can be recognized in the context of viral challenge with PR8-$HCV_{NS5B}$ virus.

### 3.4. Identification of the TCR involved in $NS5B_{2594–2602}$ recognition

A number of studies have indicated that the diversity of the TCR repertoire can reflect the avidity, quality of cytokine production and overall magnitude of the $CD8^+$ T cell response (Day et al., 2007; Messaoudi et al., 2002; Neveu et al., 2008). Because Vβ usage can provide information on the diversity and the number of $CD8^+$ T cells that can be recruited into the response, we examined the Vβ involvement of $NS5B_{2594–2602}$-specific T cells in HHD mice primed with NS5-LP and challenged 30 days later with PR8-$HCV_{NS5B}$ virus. The $NS5B_{2594–2602}$-tetramer$^+$ $CD8^+$ cells were identified from spleen cells at day 10 post-infection.

Of the seven animals examined, five mice exhibited preferential usage of the Vβ8.1/8.2 chains. The Vβ8.1/8.2 usage contributed to between 17% and 81% (mean of 39 ± 25%) of the $NS5B_{2594–2602}$-tetramer$^+$ $CD8^+$ population. A Vβ4 bias was also observed in two mice, and one mouse exhibited a Vβ10 dominant population (Fig. 3C).

To identify the TCR repertoire involved in $NS5B_{2594–2602}$ recognition, we used single cell RT-PCR to sequence the TCR CDR3β regions of the $NS5B_{2594–2602}$-specific Vβ8.1/8.2$^+$ or Vβ4$^+$ $CD8^+$ T cells from mouse 1/2/3 or mouse 4, respectively. We found that the Vβ8.1/8.2 response in mouse 1, mouse 2 and mouse 3 was comprised of four different CDR3β motifs ranging 8 to 10 amino acids long with preferred utilization of the Jβ region, 2.6 (Table 1). These mice shared a common CDR3β clonotype, SDPGGSYEQY, which was the predominant motif in mouse 1 and mouse 2 and contributed to 20% of the response in mouse 3. The Vβ4 response in mouse 4 was almost exclusively represented by a 12 amino acid long CDR3β clonotype, SQDWGSSAETLY that used the Jβ region, 2.3 (Table 1). The only other motif identified differed from the main motif by a single G-S difference at position 5. All of the identified clonotypes were encoded by a single nucleotide sequence, suggesting that each population were derived from a single T cell clone.

### 3.5. The memory phenotype of $NS5B_{2594–2602}$ specific $CD8^+$ T cells that are elicited following either vaccination or vaccination and challenge

For effective vaccination, $CD8^+$ T cell responses generated by immunization need to be recalled and deployed in the event of infection. To examine the memory phenotype of $NS5B_{2594–2602}$ specific $CD8^+$ T cells that were induced following lipopeptide vaccination, we examined the expression of the memory marker CD27 and the activation marker CD43 on $CD8^+$ T cells (Fig. 4A). Memory $CD8^+$ T cells with $CD27^{hi}CD43^{lo}$ or $CD27^{hi}CD43^{hi}$ expression have both

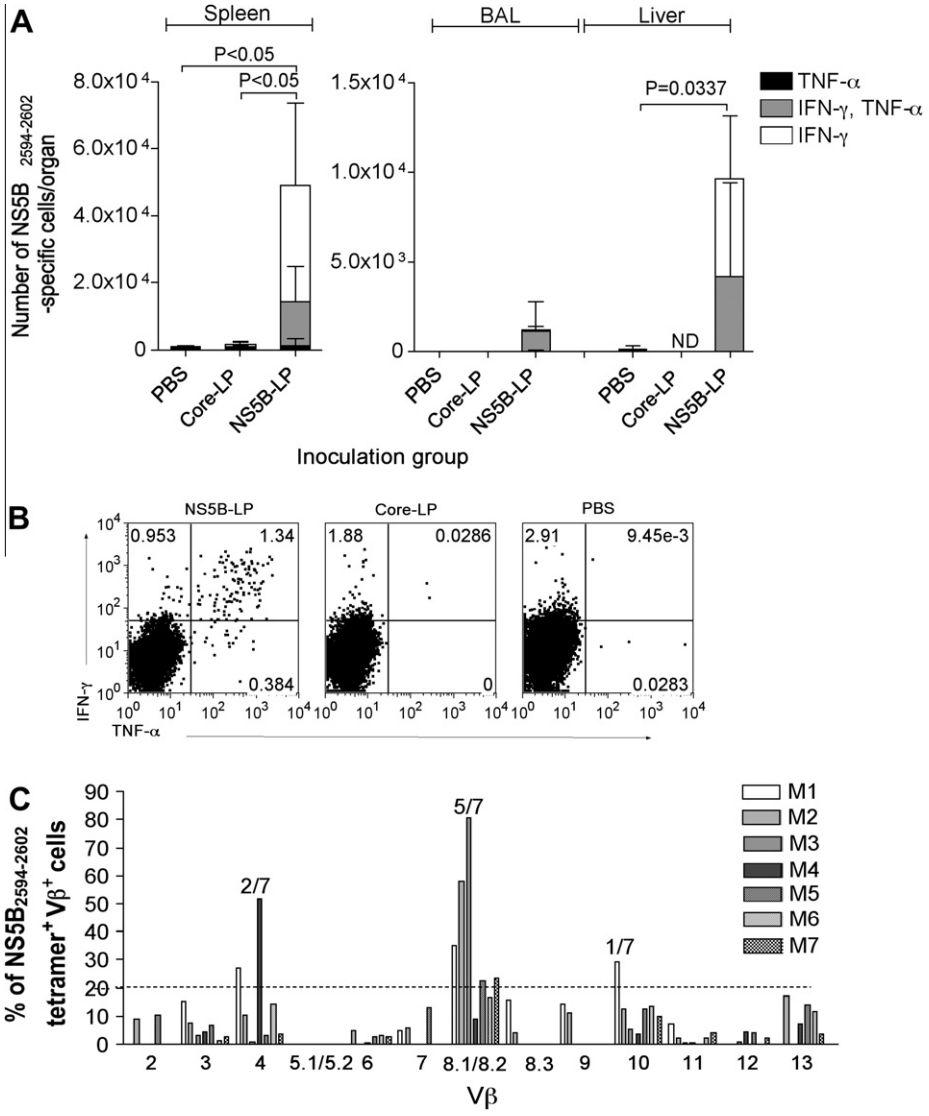


**Fig. 3.** The $NS5B_{2594–2602}$-specific response in lipopeptide-inoculated mice challenged with PR8-$HCV_{NS5B}$ virus. HHD mice were inoculated i.n. with NS5B-LP ($n = 5$), Core-LP ($n = 4$) or PBS ($n = 5$) and 2 months later mice were challenged i.n. with 200 pfu/ml PR8-$HCV_{NS5B}$ virus. On day 6 following challenge $NS5B_{2594–2602}$-specific cytokine-producing $CD8^+$ T cells were detected in the spleen, BAL fluid and liver by *ex vivo* ICS assay (A). Bars represent the mean response from each group and *P* values indicate significant differences between groups. (B) Representative FACS plots of the IFN-γ and TNF-α cytokine profile for the splenic NS5B-specific $CD8^+$ T cells in each inoculation group. Numbers indicate the percentage of cells in the respective quadrant. (C) Vβ usage in the $NS5B_{2594–2602}$-specific $CD8^+$ T cell population. $NS5B_{2594–2602}$-specific $CD8^+$ T were identified from spleen cells using $NS5B_{2594–2602}$-specific tetramers. The Vβ usage in the $NS5B_{2594–2602}$ $tetramer^{+ve}$ populations is shown for seven mice vaccinated with NS5B-LP then challenged with PR8-$HCV_{NS5B}$. The Vβ usage was considered to be biased when the percentage of $V\beta^+$ cells exceeded 20% and the values above the bars indicate the portion of mice exhibiting Vβ bias.

**Table 1**
Frequency of TCRβ amino acid sequences in $NS5B_{2594–2602}$-$tetramer^+$ $CD8^+$ T cells.

| | | | | Frequency of sequences (%) | | | |
|---|---|---|---|---|---|---|---|
| CDR3β | Vβ | Jβ | Length | M1 | M2 | M3 | M4 |
| SDGTGDSDYT | 8.1 | 1.2 | 10 | | | 77 | |
| SDLGGAEQF | 8.1 | 2.6 | 9 | | | 2 | |
| SDPGGSYEQY | 8.1 | 2.6 | 10 | 98 | 95 | 21 | |
| WGGSYEQY | 8.1/8.2 | 2.6 | 8 | 2 | 5 | | |
| SQDWGSSAETLY | 4 | 2.3 | 12 | | | | 98 |
| SQDWSSSAETLY | 4 | 2.3 | 12 | | | | 2 |
| Total # of sequences | | | | 41 | 44 | 57 | 44 |

M: an individual mouse.

been reported to be effectively recalled following infection or vaccination (Hikono et al., 2007).

Using intracellular cytokine staining we identified IFN-γ producing $NS5B_{2594–2602}$ specific $CD8^+$ T cells that were induced by

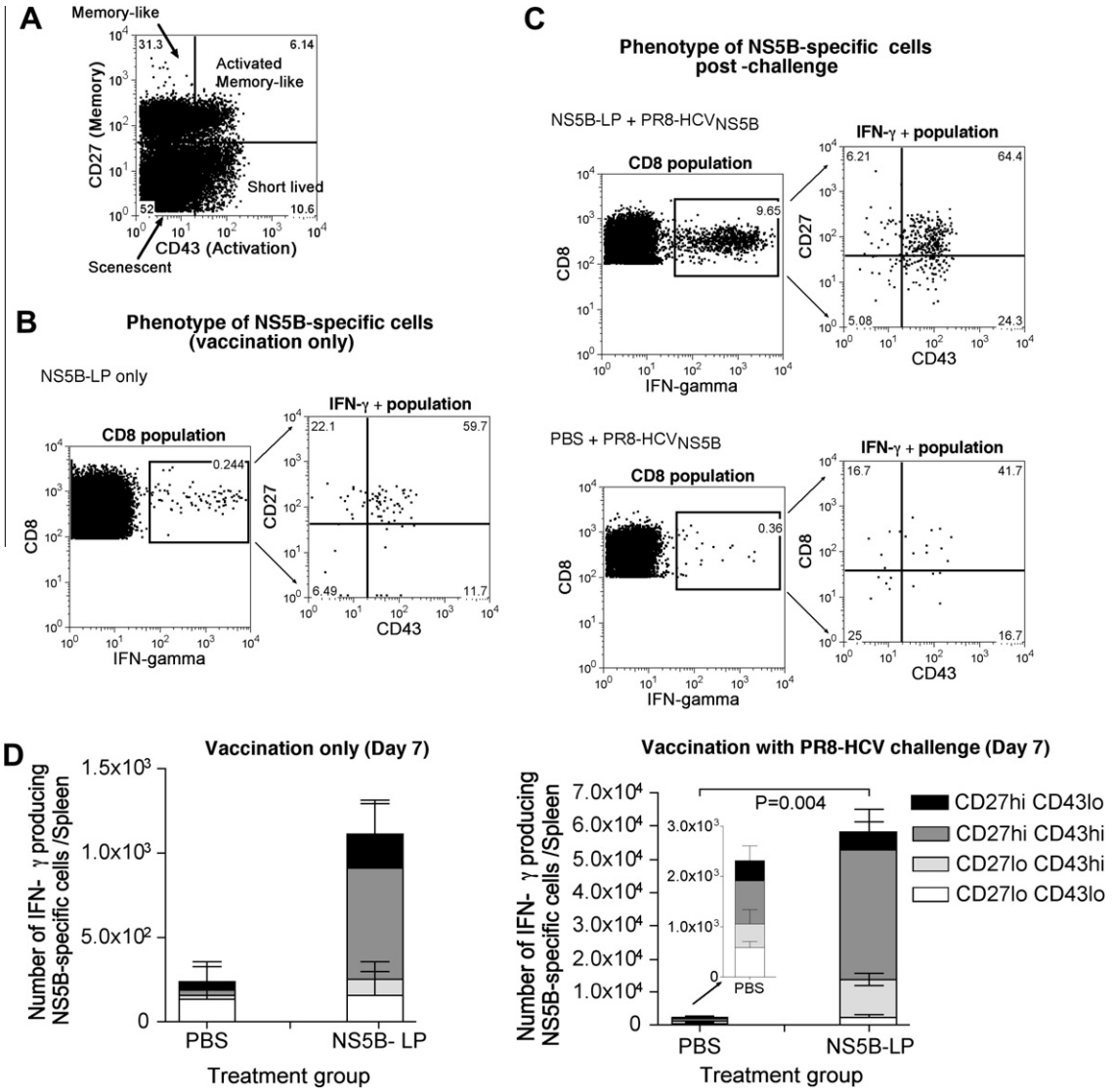


**Fig. 4.** The memory activation phenotype of $NS5B_{2594–2602}$-specific $CD8^+$ T cells induced by vaccination or vaccination and virus infection. HHD mice were either vaccinated i.n. with NS5B-LP or PBS or vaccinated and challenged i.n. 1 month later with 200 pfu/ml PR8-$HCV_{NS5B}$ virus. Seven days after vaccination or vaccination with challenge, the splenic NS5B-specific $CD8^+$ T cells were identified by IFN-γ detecting intracellular cytokine staining assay. (A) The quadrant gates used to segregate different memory $CD8^+$ T cell sub-populations based on memory (CD27) and activation (CD43) expression is shown for a representative total $CD8^+$ T cell population. The numbers represent the percentage of cells in the gates. (B) In NS5B-LP vaccinated mice, FACS charts illustrate the splenic IFN-$\gamma^+$ NS5B-specific $CD8^+$ T cells (left chart) and their predominantly $CD27^{hi}CD43^{hi}$ phenotype (right chart). (C) FACS charts illustrate the IFN-$\gamma^+$ NS5B-specific $CD8^+$ T cells (left chart) and CD27 versus CD43 phenotype (right charts) in mice following PR8-$HCV_{NS5B}$ viral challenge. (D) The subpopulations of the IFN-$\gamma^+$ NS5B-specific cells based on CD43 and CD27 expression is shown for mice that received vaccination only (left) or vaccination plus PR8-$HCV_{NS5B}$ challenge (right).

NS5B-LP vaccination and demonstrated that the phenotype of these cells was predominantly $CD27^{hi}CD43^{hi}$ (Fig. 4B and D). Because the CD27 CD43 phenotype of IFN-γ only and IFN-γ TNFα co-producing cells were similar, the *total* IFN-γ positive $NS5B_{2594–2602}$ specific $CD8^+$ T cells response is shown in Fig. 4 for simplicity.

In order to compare the memory $CD8^+$ T cell phenotype in mice that were challenged with PR8-$HCV_{NS5B}$ only (i.e. the PBS group) with those which received NS5B-LP prior to challenge, we examined responses at day 7 post-challenge, a time point at which the PBS group would have established $CD8^+$ T cell responses to infection. Following challenge the animals that received lipopeptides exhibited significantly and substantially higher numbers of $NS5B_{2594–2602}$ specific-$CD8^+$ T cells compared to the PBS group. These cells predominantly expressed the activated memory-like $CD27^{hi}CD43^{hi}$ phenotype. Moreover, this phenotype was similar to that detected in the PBS group (Fig. 4C and D) indicating that lipopeptide vaccination primes memory $CD8^+$ T cells with a high recall capacity and phenotype similar to that expected to follow natural infection.

### *3.6. NS5B-LP inoculation reduces viral burden, in a manner that is associated with IFN-γ and TNF-α producing $CD8^+$ T cells*

Previous studies have found IFN-γ and TNF-α production to be associated with viral clearance, often in a synergistic manner (Chen et al., 1993; Guidotti et al., 1996; Neveu et al., 2008; Pavic et al., 1993; Phillips et al., 2010). We have found that following lipopeptide-priming and subsequent challenge with the recombinant virus resulted in IFN-γ and TNF-α-producing $NS5B_{2594–2602}$-specific $CD8^+$ T cells. To assess the protective capacity of NS5-LP inoculation, we measured the viral load in the PR8-$HCV_{NS5B}$-challenged

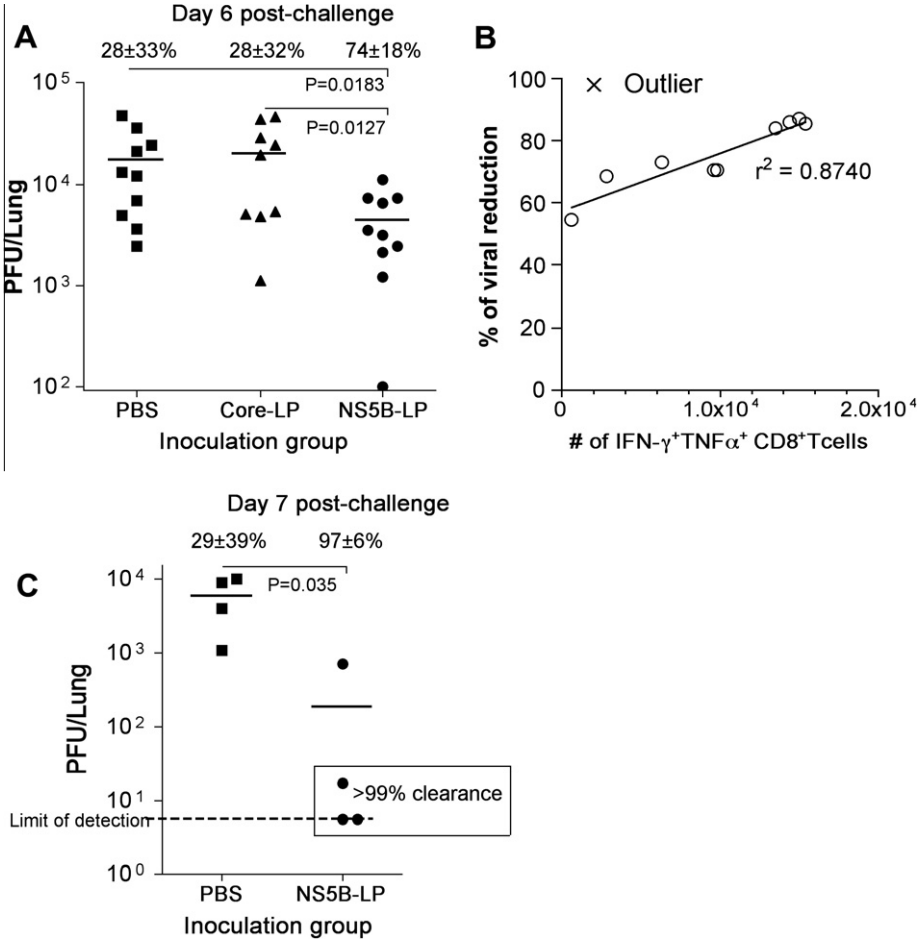


**Fig. 5.** Polyfunctional NS5B-specific CD8$^+$ T cells are associated with viral reduction. HHD mice inoculated with NS5B-LP, Core-LP or PBS were challenged 2 months later with 200 pfu/ml PR8-HCV$_{NS5B}$. The lung viral load at day 6 post-challenge is shown in (A). The values for the reduction in viral load relative to the PBS treatment group are shown above each data set. The number of splenic IFN-γ TNF-α co-producing cells is plotted against the viral reduction for each individual mouse in the NS5B-LP groups (B). The linear regression ($P = 0.0002$) is indicated with a line and the $r^2$ value is shown. An outlier is indicated by the × symbol. In a separate experiment, the lung viral load was assessed at day 7 post-challenge and the viral titers are shown in (C). The results in A are from a single experiment that was representative of two separate experiments. Statistical differences between viral titers were calculated by an unpaired student's *t*-test. The linear regression in B combined the result from these two experiments ($n = 9$) and excludes the outlier.

mice that had been previously inoculated with NS5B-LP. At day 6 following challenge all mice showed evidence of PR8-HCV$_{NS5B}$ infection, however, mice inoculated with NS5B-LP exhibited significantly lower lung viral loads than animals that received the control lipopeptide Core-LP ($P = 0.0127$) or PBS ($P = 0.0183$) (Fig. 5A). When the relationship between cytokine producing capacity of the NS5B$_{2594–2602}$-specific CD8$^+$ T cells and viral reduction was compared, we found a positive correlation ($r^2 = 0.8740$) between the number of CD8$^+$ T cells producing both IFN-γ and TNF-α and the level of viral reduction observed (Fig. 5B). A single mouse did not follow this pattern and is shown as an "outlier" and was therefore not included in the regression analysis. This mouse displayed a low viral titer in the absence of a strong CD8$^+$ T cell response indicating that it may have been improperly infected. In contrast, the correlation coefficients ($r^2$) between the percentages in viral reduction and the numbers of CD8$^+$ T cells producing IFN-γ only and TNF-α only were 0.057 and 0.3887, respectively (data not shown). This suggests that in this infection model, it is the polyfunctional T cells that are mostly associated with viral reduction.

In order to determine whether vaccination could accelerate viral clearance in addition to reducing viral loads, we measured the viral burden in NS5B-LP vaccinated mice 7 days after challenge. We found that the majority of mice vaccinated with NS5B-LP exhibited almost complete (>99%) clearance of virus when compared to the PBS control (Fig. 5C). We also noted that mice demonstrating >99% viral clearance possessed a twofold higher number of IFN-γ$^+$ and TNF-α$^+$ cells in their lungs when compared to mice demonstrating 88% clearance (data not shown). These results are consistent with the observation that this phenotype is important for viral control and clearance.

We also examined the efficacy of NS5-LP inoculation against PR8-HCV$_{NS5B}$ challenge in a different model using HLA-A2/K$^b$ transgenic mice. The mice express a recombinant form of HLA-A2 on a C57BL/6 background. Unlike the HHD mice, HLA-A2/K$^b$ mice also express endogenous mouse class I H2D$^b$ molecule and these mice have been found to exhibit substantially lower HLA-A2-restricted responses than HHD mice (Firat et al., 1999, 2002). In HLA-A2/K$^b$ mice inoculated with two doses of NS5B-LP, however, we detected NS5B$_{2594–2602}$-specific epitopes in restimulated spleen cells by an IFN-γ detecting ELISPOT assay (Supplemental Fig. S3A). Mice inoculated with NS5B-LP and then challenged 30 days later with 50 pfu of PR8-HCV$_{NS5B}$ exhibited significantly lower viral loads than mice inoculated with PBS or a control lipopeptide containing an HSV-1-derived epitope (HSVgB-LP) (Fig. S3B). These data reflects the findings obtained in the HHD mice, and support a role for NS5B-LP-based lipopeptide inoculation

and CD8$^+$ T cell activity in enhancing the clearance of a recombinant virus containing an HCV epitope.

## 4. Discussion

Many studies have indicated a role for CD8$^+$ T cells in the control and clearance of HCV infection, prompting the quest to develop a therapeutic CD8$^+$ T cell vaccine for HCV treatment.

In this study, we have demonstrated the immunogenicity of intranasally delivered HCV epitope-based lipopeptides. The lipopeptide mixture could induce up to four different HCV peptide-specific responses when delivered simultaneously and importantly could induce polyfunctional cytokine-producing CD8$^+$ T cells in multiple organs including the liver. By adopting the intranasal route of inoculation, we successfully induced *de novo* functional liver-resident CD8$^+$ T cells. This suggests that lipopeptide vaccination at a site *distal* to that being targeted, such as the lung which is highly conducive to CD8$^+$ T cell priming, may be useful when responses are desired in organs such as the liver which are otherwise poorly-conducive to the priming of effector CD8$^+$ T cells (Crispe et al., 2000; Holz et al., 2010). Although peptide-based vaccination has had limited success in the clinic, recent clinical trials with the synthetic peptide-based vaccine, IC41, containing the T cell adjuvant poly-L-arginine have shown promising results in chronically infected patients (Klade et al., 2009). This particular vaccine utilizes five different CD4$^+$ and CD8$^+$ HCV epitopes which elicited broad T cell immunity and significantly reduced viremia when delivered via the less conventional, intradermal route (Klade et al., 2009). These results substantiate the potential benefits of peptide-based vaccination in a therapeutic setting and emphasize the importance of selecting an appropriate delivery route to achieve vaccine efficacy.

In the present study we found that multi-lipopeptide vaccination induced responses with a marked immunodominance hierarchy. Because the PR8-HCV$_{NS5B}$ virus model contains a single epitope only, we were unable to study the impact of this hierarchy on vaccine efficacy in the context of viral challenge. We have, however, previously shown (Tan et al., 2011) that the hierarchy of the CD8$^+$ T cell response during vaccination and infection is dictated by the naïve CD8$^+$ T cell precursor frequency. More recently, the naïve CD8$^+$ T cell precursor frequency has been shown to affect the hierarchy of HCV-specific CD8$^+$ T cell responses (including those to NS3$_{1406–1415}$ and NS5B$_{2594–2602}$) in HCV-infected individuals (Schmidt et al., 2011). In contrast to what has been observed with influenza A specific responses, the HCV-specific CD8$^+$ T cell hierarchy observed by Schmidt et al. (2011) differed from the CD8$^+$ T cell hierarchy that we detected in HHD mice. This suggests that there may be (for at least some specificities of CD8$^+$ T cells) differences between the naïve CD8$^+$ T cell populations between HHD mice and HLA-A2$^+$ humans. Nevertheless, it is likely that the immunodominance hierarchy we detected to HCV lipopeptide vaccination reflects the inherent capacity in these mice to respond to these epitopes and, would be maintained in the event of a HCV infection (if a permissive infection was available).

We found the TCR repertoire diversity within the Vβ8.1/2 and Vβ4 segments of the NS5B$_{2594–2602}$-specific responses to be narrow, but "public", suggesting that at least in HHD mice there is enlistment of a limited range of "best fit" clonotypes into the response. A lack of TCR diversity is not thought to impede the functionality of the CD8$^+$ T cells (La Gruta et al., 2008); however, narrow TCR repertoires are proposed to be less able (than diverse repertoires) to offer promiscuous cross-recognition to variant or mutant epitopes and may, in fact, drive the appearance of immune driven escape mutants (Meyer-Olson et al., 2004; Price et al., 2004). The persistent nature of chronic HCV infection is likely to exert pressure towards immune escape as has been reported for the HCV NS3$_{1406–1505}$ epitope (Wolfl et al., 2008). This highly immunodominant CD8$^+$ T cell target has been shown to escape recognition by taking advantage of a "hole" in the TCR repertoire to avoid detection by pre-existing NS3$_{1406–1505}$ CD8$^+$ T cells (Wolfl et al., 2008). It is not yet known whether the NS5B$_{2594–2602}$-specific TCR repertoire identified in HHD mice reflects the repertoire found at the human population level and to what extent the identified NS5B$_{2594–2602}$-specific mutants (Kasprowicz et al., 2010; Tester et al., 2005) impact on CD8$^+$ T cell responses and clinical outcome. In a recent observation, a narrow mono-specific CD8$^+$ T cell response was associated with persistent viremia and poor disease outcome (Tester et al., 2005). This reiterates the importance of targeting multiple CD8$^+$ T cell specificities by vaccination to ensure that there is a broad response available to compensate for any losses in CD8$^+$ T cell subpopulations due to epitope escape. Therefore it is almost certain that future vaccines will need to deliver multiple epitopes to be effective against HCV infection. As lipopeptides can be readily modified to include different epitopes in polytope form (Torresi et al., 2007) or through conjugation of Pam2Cys to whole proteins (Chua et al., 2011; Zeng et al., 2011) they can be readily designed to elicit a broad array of CD8$^+$ T cell responses and thereby address issues associated with epitope escape and narrowing of the response.

To examine the efficacy of lipopeptide vaccination, we developed a recombinant influenza HCV virus, PR8-HCV$_{NS5B}$ expressing the NS5B$_{2594–2602}$ epitope to study the immune responses of animals inoculated with an HCV lipopeptide vaccine candidate. Although the profile of influenza A virus infection differs from that of hepatitis C, influenza-vectors can be readily used in laboratory mice and offer increased user safety over other HCV models such as the vaccinia-based viruses (Eisenbach et al., 2007). Using the recombinant PR8-HCV$_{NS5B}$ virus, we demonstrated that prophylactic vaccination with the NS5B$_{2594–2602}$ epitope-based lipopeptide significantly enhanced the clearance of the PR8-HCV$_{NS5B}$ recombinant virus in HHD mice. Although the lipopeptide-induced CD8$^+$ T cells did not prevent infection, this is in line with the role of CD8$^+$ T cells in the clearance of established viral infection, and not prevention of influenza infection (Graham and Braciale, 1997). Moreover, the antiviral activity was associated with the presence of IFN-γ/TNF-α co-producing NS5B$_{2594–2602}$-specific CD8$^+$ T cells. In contrast, when Vβ usage and viral reduction was compared, we found no apparent correlation between the extent of the Vβ8.1/8.2 bias (or the number of Vβ8.1/8.2$^+$ NS5B$_{2594–2602}$-tetramer$^+$ CD8$^+$ T cells) and the level of viral clearance observed in individual mice. These findings suggest that cytokine production is a better predictor for CD8$^+$ T cell mediated viral clearance than either Vβ bias, TCR repertoire diversity or the number of tetramer positive cells.

An association between the presence of polyfunctional high-avidity-IFN-γ TNF-α producing (Neveu et al., 2008) or IFN-γ IL-2 producing CD8$^+$ T cells (Badr et al., 2008; Ciuffreda et al., 2008) with early viral control and improved outcome in HCV-infected patients has been previously reported. Our current findings suggest that polyfunctional CD8$^+$ T cell populations are important for the clearance of the recombinant virus PR8-HCV$_{NS5B,}$ supporting this profile of T cells in vaccine-mediated antiviral activity. This relationship between CD8$^+$ T cell cytokine profile and viral control has been likewise reported in a study using a vaccinia-based HCV surrogate challenge model in mice (Mikkelsen et al., 2011).

An important consideration in the development of CD8 vaccines against HCV, is the concern that HCV-specific CD8$^+$ T cells contribute to liver fibrosis and immune-mediated liver pathology (Nelson et al., 1997; Neuman et al., 2001). Until a direct HCV or hepatic challenge model is made available for study in immune competent mice, we cannot predict the outcome of lipopeptide-induced CD8$^+$ T cell activity on liver pathology in a therapeutic setting. However,

a study carried out in chimpanzees inoculated with HCV-virus like particles expressing core, E1 and E2 proteins, found that $CD4^+$ and $CD8^+$ T cells activity was associated with reduced viremia and prevention of persistent infection in the *absence* of liver injury. These results suggest that vaccine-induced cell-mediated immunity does not necessarily exacerbate liver pathology (Elmowalid et al., 2007) and may rely on non-cytolytic mechanisms of activity (Thimme et al., 2002).

## 5. Conclusion

We have shown that lipopeptide-based vaccines when delivered intranasally are effective in inducing polyfunctional $CD8^+$ T cells directed at HCV-specific epitopes, demonstrating their potential for development as an alternative treatment option for HCV. The observation that the polyfunctional $CD8^+$ T cells are important in viral clearance also identifies a $CD8^+$ T cell phenotype that could provide a measure of effectiveness in future vaccine strategies.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2012.03.009.

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