Journal of Immunological Methods 472 (2019) 65–74

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

A method for detecting hepatitis C envelope specific memory B cells from multiple genotypes using cocktail E2 tetramers

Bing-Ru Wu, Auda A. Eltahla, Elizabeth Keoshkerian, Melanie R. Walker, Alex Underwood, Nicholas A. Brasher, David Agapiou, Andrew R. Lloyd[1], Rowena A. Bull[*,1]

School of Medical Sciences and the Kirby Institute, Faculty of Medicine, UNSW Australia, Sydney, NSW 2052, Australia

ARTICLE INFO

Keywords:
HCV
E2-specific B cells
Tetramer
Monoclonal antibodies and hybridoma cells

ABSTRACT

Hepatitis C (HCV) is a rapidly mutating RNA virus, with a strong propensity to cause chronic infection and progressive liver disease. Recent evidence has shown that early appearance of neutralizing antibodies in primary infection is associated with clearance. Little is known about the characteristics of HCV-specific B cells and their correlation with outcomes in primary infection, as there is a lack of sensitive tools for HCV-specific B cells which are present at very low frequency. We describe the development and optimisation of tetramer staining for flow cytometric detection of HCV-specific B cells using a cocktail of two recombinant HCV Envelope-2 (rE2) glycoproteins (from genotype 1a and 3a; Gt1a and Gt3a) and streptavidin dyes. The optimal weight to weight (*w*/w) ratio of streptavidin-phycoerythrin (PE) and rE2 proteins were determined for sensitive detection using HCV E2-specific hybridoma cell lines and peripheral blood mononuclear cells (PBMC) from HCV-infected individuals. In a cross-sectional set of PBMC samples collected from 33 subjects with either chronic infection or previous clearance, HCV E2-specific B cells ($CD19^+CD20^+CD10^-IgD^-tetramer^+$) were detected in 29 subjects (87.8%), with a mean frequency of 0.45% (0.012–2.20%). To validate the specificity of tetramer staining, 367 HCV E2-specific B cells were single cell sorted from 9 PBMC samples before monoclonal antibodies (mAbs) were synthesised, with 87.5% being reactive to E2 via ELISA. Of these mAbs, 284 and 246 clones were reactive to either Gt1a or Gt3a E2 proteins, respectively. This is a sensitive and robust method for future studies investigating B cell responses against the HCV Envelope protein.

1. Introduction

Hepatitis C virus (HCV) infection is a leading cause of chronic liver disease, cirrhosis and hepatocellular carcinoma (HCC) worldwide. Recent surveillance data suggest that in 2015, there were 71 million individuals worldwide who were chronically infected with HCV and 1.75 million new cases annually (Polaris Observatory, 2017; Hoshida et al., 2014). Although direct acting antivirals (DAAs) have proved to be highly effective in the treatment of chronic HCV, uptake remains limited by high cost and limited health infrastructure. In addition, cure does not protect against reinfection (Falade-Nwulia et al., 2018). Thus, the development of a prophylactic vaccine against HCV transmission is a major strategic goal in reducing the burden of HCV disease.

It is generally believed that a vaccine that can induce sterilising immunity against HCV will be challenging to develop due to the large genetic diversity across the eight HCV genotypes (Borgia et al., 2018). As the major health burden of HCV arises from the majority of patients who fail to spontaneously clear the virus during primary infection, a vaccine that can facilitate clearance would be highly effective in reducing the disease burden (Scott et al., 2015). Antiviral immune responses involving $CD4^+$ and $CD8^+$ T cells, as well as neutralizing antibodies, have been shown to be associated with viral clearance in primary infection (Shoukry, 2018), but only two studies have examined HCV-specific B cell responses (Boisvert et al., 2016; Umemura et al., 2006). The earlier study used a memory B cell ELISpot assay in samples from patients with chronic infection and identified antigen-stimulated immunoglobulin secretion in the majority of subjects. The more recent study used a tetramerized genotype (Gt) 2 HCV Envelope protein probe to bind to antigen-specific B cells via the B cell receptor (BCR) for detection in flow cytometry, a method established for study of antigen-specific B cells against several other pathogens such as HIV, dengue and influenza viruses (Moody and Haynes, 2008; Degauque et al., 2013; Sundling et al., 2012). In this method, a tetramerized fluorescent probe conjugated to viral particles or viral proteins, binds to the surface BCR

* Corresponding author.
E-mail address: r.bull@unsw.edu.au (R.A. Bull).
[1] These authors contributed equally.

https://doi.org/10.1016/j.jim.2019.06.016
Received 14 March 2019; Received in revised form 13 June 2019
Available online 19 June 2019

enabling detection. Careful consideration of both how well the labelling protein resembles the native structure and the ease-of-use needs to be considered when selecting the approach.

In the case of HCV, the proteins of greatest interest for B cell research are the two Envelope glycoproteins 1 (E1) and 2 (E2), as they facilitate cell entry and are the primary target of neutralizing antibodies (Bartosch et al., 2003; Douam et al., 2014). Unfortunately, the E1E2 heterodimer contains large hydrophobic transmembrane domains preventing reliable generation of soluble E1E2 (Ciczora et al., 2007). Recently, truncated forms of soluble E1E2 and E2 have been generated, with several different variants of E2 being routinely generated by many groups (Carlsen et al., 2013; Maurin et al., 2011). The recent report of staining with an HCV Envelope tetramer used recombinant HCV E2 (rE2) protein from a unique Gt2a strain (Boisvert et al., 2016), which is infectious in cell culture (Lindenbach et al., 2005; Khan et al., 2014). Unfortunately Gt2 only represents 11% of the global burden of HCV infection, whereas Gt1 and Gt3 are the most common, representing 49% and 18% of all infections globally (Petruzziello et al., 2016). Due to the large genetic variation (approximately 30% nucleotide divergence) between HCV genotypes, the choice of probe is very likely to influence the sensitivity in detection of HCV-specific B cells (Simmonds et al., 2005). A previous attempt to make a Gt1 tetramer was unsuccessful (Boisvert et al., 2016).

Here, we report successful development and optimisation of HCV Gt1a and Gt3a rE2 tetramers that can either be used separately, or in combination, to analyse B cells via flow cytometry. Single cell sorting and production of monoclonal antibodies (mAbs) confirmed the assay to be highly sensitive for E2-specific B cells. The rE2 tetramers developed in this study offer a valuable tool for studying the kinetics and phenotype of memory B cells generated during HCV infection.

2. Materials and methods

2.1. Study participants

Blood samples were collected from subjects enrolled in two prospective cohorts of HCV seronegative, high-risk individuals who reported injecting drug use (IDU) - the Hepatitis C Incidence and Transmission Study (HITS) in prisons (HITS-p) and the community (HITS-c) between 2005 and 2014 (Luciani et al., 2014; Teutsch et al., 2010; Dolan et al., 2010). Participants were recruited from 23 correctional centers across New South Wales, Australia (HITS-p) and six Sydney neighbourhoods located in three distinct regions across greater metropolitan Sydney (HITS-c). A total of 33 subjects were examined in the cross-sectional analysis reported here, including 17 subjects who had cleared previous infection ('clearers' whose samples were HCV antibody positive and RNA negative) and 16 who became chronically infected ('chronics' whose samples were HCV antibody positive and RNA positive). The median estimated days post-infection (DPI) was 507 days (range, 215–803 DPI) for the clearers and 543 days (range, 378–2109 DPI) for the chronics (Table 1). Samples from a group of low risk, HCV-uninfected, healthy blood donors were obtained from the Australian Red Cross Blood Service (n = 10) for use as negative controls.

Table 1
Clinical characteristics of the HCV cohort.

Clinical characteristic	Clearers (n = 17)	Chronics (n = 16)
Infecting genotype		
Gt1	6	9
Gt3	7	7
Gt1/3	3	0
Gt3/6	1	0
Gender (M/F)	9/8	10/6
Estimated days post-infection; median (range)	507 (215–803)	543 (378–2109)

2.2. Ethics

Ethical approval was obtained from the Human Research Ethics Committee of Justice Health (reference number G304/11), New South Wales Department of Corrective Services (11/103694) and the University of New South Wales (HREC HC11579 and HREC HC13237). Written informed consent was obtained from all participants.

2.3. Construction and purification of biotinylated recombinant E2

Recombinant E2 (rE2) glycoproteins were constructed for the two most prevalent HCV genotypes in Australia and worldwide – Gt1 and Gt3 (Walker et al., 2016). The rE2 protein sequence was derived from HCV H77 Gt1a (GenBank ID: AF011751) and Gt3a (AY894683). E1E2 plasmids were kindly gifted by Jonathan Ball (Tarr et al., 2007). The rE2 construct incorporated the HCV polyprotein amino acid residues 384 to 661 into a pcDNA3.1 expression vector (ThermoFisher Scientific, Waltham, MA, USA) with a N-terminal secretion signal from the V-J2-C region of the mouse IgG-chain and a C-terminal Avitag™ and an added six-Histidine epitope tag (6× His) (Wang et al., 2011; McCaffrey et al., 2017). This codon optimized construct was purchased from GeneArt (ThermoFisher Scientific, Waltham, MA, USA). Transient transfection of expression plasmids was performed using FreeStyle 293-F cells using DNA-293fectin reagent as per the manufacturer's instructions (ThermoFisher Scientific, Waltham, MA, USA). Media was then harvested at approximately 96 h post-transfection for purification. This supernatant was then passed through a 1 ml HiTrap chelating HP chromatography column (GE Healthcare, Uppsala, Sweden) charged with 0.1 M $NiSO_4 6H_2O$ and washed with 10 ml binding buffer. Proteins were eluted from the column by passing through 50 mM imidazole in binding buffer. Elution fractions were confirmed using SDS-PAGE and western blot. Site-specific biotinylation of the rE2 was performed using the BirA biotin-protein ligase standard reaction kit (Avidity, LLC, Aurora, CO) and antibody binding efficacy confirmed using ELISA and western blot.

2.4. Production of cocktail tetramer and validation on HCV E2-specific hybridoma cell lines (CBH-5)

The following HCV rE2 tetramerization method was adapted from the MHC Class I CD8 tetramer preparation protocol from the NIH Core Tetramer facility (Facility, 2018). E2 glycoprotein are present in dimeric and monomeric forms, therefore tetramers were prepared based on weight-to-weight ratios of streptavidin dye and E2 protein rather than molar ratios (Flint et al., 2000). In brief, Streptavidin-PE (SA-PE; Molecular Probe; ThermoFisher Scientific, Waltham, MA, USA) was incubated with either biotinylated (b) Gt1a, Gt3a or Gt1a/3a rE2 (brE2) cocktails (represented as cocktail tetramers; Gt1a/3a rE2 mixed at a 2:1 w/w ratio) at a ratio of SA-PE:brE2 at a 1:2.5 w/w ratio (eg. 10 μg:25 μg). Streptavidin-dye was titrated step-wise in 1/10th volumes to the brE2 for a total of 10 times, followed by an incubation of 10 min at 4 °C in a rotating bioreactor, protected from light. Before staining, either Gt-specific or cocktail tetramers were centrifuged at 21,000g for 10 min to remove protein aggregates. Prior to tetramer staining, 1×10^6 CBH-5 hybridoma cells (obtained from ATCC PTA-4469) were stained with 1:10,000 dilution of fixable red viability stain (BD Biosciences, San Jose, CA, USA) to distinguish live and dead cells. The hybridoma cells were washed twice with washing buffer (1% [w/v] BSA in PBS) and then stained with either Gt-specific, or cocktail tetramers for 30 min at 4 °C. Cells then were stained with 5 μl of anti-CD3-APC H7 and 10 μl of anti-IgG-BV786 (BD Biosciences) for 30 min at 4 °C. Those two antibodies were used to identify non-T cells and surface E2-specific IgG, respectively. Cells were then washed twice with washing buffer before being fixed in 1% paraformaldehyde (Sigma-Aldrich, St. Louis, MO).

2.5. PBMC staining and flow cytometry analysis

Cryopreserved PBMCs were thawed and washed with pre-warmed RPMI medium supplemented with 2 mM L-glutamine, 50 IU/ml penicillin, 50 μg/ml streptomycin and 10% heat inactivated foetal calf serum (Life Technologies; ThermoFisher Scientific, Waltham, MA, USA) and subsequently washed with PBS buffer prior to further staining. A maximal 10×10^6 cells were then stained with fixable red viability stain (BD Bioscience, 1:1000 dilutions) and incubated at 4 °C for 30 min to identify dead cells. Cells were then washed twice with washing buffer, followed by the incubation of 5 μl Human Fc block/2×10^6 cells (BD Bioscience, San Jose, CA, USA) at room temperature for 10 min to block possible non-specific antibody binding. Meanwhile, cocktail tetramers were centrifuged at 21,000 *g* for 10 min before staining of the PBMCs. HCV E2-specific B cells were identified after staining with 7.5 μg of cocktail tetramers at 4 °C for 30 min, protected from light. Immunophenotyping for HCV E2-specific B cell subsets was performed using staining buffer containing 50 μl stain brilliant buffer (BD Bioscience, San Jose, CA, USA) and the titrated combination of antibodies: 2 μl of anti-CD10-BV605, 2 μl of anti-CD20-APC H7, 5 μl of anti-IgD-BV510, 5 μl of anti-CD19-BV711 and 2 μl of anti-CD27-PE CF594 (all from BD Biosciences). FACS analyses and sorting were performed on a FACSAria II flow cytometer (BD Biosciences), with data analyses performed using FlowJo version 10 software (Tree Star Inc., Ashland, OR).

2.6. Production of monoclonal antibodies from single-sorted HCV E2-specific B cells

Natively paired heavy and light chain variable (V_H and V_L) region sequences were obtained by single cell RNAseq and used to determine specific primers for amplification and subsequent recombinant Ab expression of natively paired V_H and V_L genes. In brief, single sorted HCV E2-specific B cells ($CD19^+CD20^+CD10^-IgD^-$tetramer$^+$) were collected into 96-well PCR plates that contained in a final volume of 2 μl per well: 0.5 μl of dNTP (10 mM) (ThermoFisher Scientific, Waltham, MA, USA), 0.5 μl of 5 μM oligo-dT primer and 1 μl of lysis buffer, lysis buffer was prepared by addition of 1 μl (40 U) RNAse inhibitor (Clontech, Mountain View, CA) to 19 μl Triton X-10 (0.2% [*v*/v]. These samples were then RT-PCR amplified with the SmartSeq 2 approach, sequenced with Illumina 2×150 PE Nextera XT Library Preparation Kit and the BCR sequences reconstructed with VDJpuzzle 2.0, as previously described (Rizzetto et al., 2018).

Unique recombinant antibodies were generated using the V_H and V_L of these single sorted E2-specific B cells, using a modified version of a previously described protocol (Liao et al., 2009). In brief, PCR amplification was performed on these variable fragments using the Platinum *Taq* DNA Polymerase kit (Life Technologies; ThermoFisher Scientific, Waltham, MA, USA) by combining 1 μl each of forward and reverse primer, 1 μl of single-sorted B cell template, and 47 μl of master mix per reaction, prepared according to the manufacturer's instructions. Primers were selected according to the previously determined heavy or light chain variable subtype, with the heavy and light chain variable fragments of each unique B cell being amplified in separate reactions (Liao et al., 2009). The PCR product was purified using Agencourt AMPure XP magnetic beads (Beckman Coulter Inc. Pasadena CA, USA) with product quality verified by gel electrophoresis. An overlapping PCR was then performed to assemble the heavy or light chain expression cassettes using the iProof HF DNA Polymerase kit (Bio-Rad, Hercules, USA) as previously described (Liao et al., 2009).

Antibodies were then generated by co-transfecting HEK293T cells with 1 μg each of heavy and light chain expression cassette using 20 μl of Polyfect Transfection Reagent (Qiagen, Hilden, Germany) and 600 μl of DMEM supplemented with 2 mM L-glutamine, and 10% heat inactivated foetal calf serum. Cells were incubated at 37 °C and 5% CO_2 for 6–8 h before media was replaced with 3 ml of DMEM supplemented with 2% heat inactivated foetal calf serum and 1% penicillin/streptomycin) and incubated under the same conditions for a further 67–72 h. Media was then collected and centrifuged to isolate supernatant containing antibodies, before storage at −20 °C.

A rE2 binding ELISA using HCV Gt1a (H77) and Gt3a (UKN3A13.6) was performed to validate the specificity the E2-specific B cell detection and resultant mAb production as previously described (Underwood et al., 2018). In brief, Nunc immuno-microtitre plates (ThermoFisher Scientific, Waltham, MA, USA) were pre-coated with 500 ng of *Galanthus nivalis* lectin (GNA; Sigma-Aldrich, St. Louis, MO, USA) before blocking with 5% skim milk (ThermoFisher Scientific, Waltham, MA, USA). Pre- and post-biotinylated HCV rE2 from Gt1a and Gt3a were captured by GNA onto the plate and later bound by either mAbs produced from patient-derived single-sorted HCV E2-specific B cells or well characterized HCV E2-sepcific mAbs, including AR2A and AR3A, which were kindly provided by Prof. Mansun Law (The Scripps Research Institute, La Jolla, California, USA), CBH-4G and CBH-5 (domain B) and CBH-7 (domain C). These antibodies were purified from supernatants of hybridoma cell lines obtained from ATCC (PTA-4468, PTA-4469, and PTA-4470, respectively). Antibodies HC84.26 (domain D), and HCV1 (domain E) were generated by transient transfection of FreeStyle™ 293-F cells with plasmids encoding heavy and light chains which were constructed as described previously (Alhammad et al., 2015). The bound mAb was detected by incubation with alkaline phosphatase-conjugated goat anti-human IgG (Jackson ImmunoResearch, West Grove, PA, USA), followed by incubation with 3,3′,5,5′-tetramethylbenzidine (TMB; ThermoFisher Scientific) for color development. Absorbance was measured at 405 nm and 570 nm.

2.7. Statistics

Comparison of HCV E2-specific B cells between the two outcome groups of HCV-infected subjects were analyzed using the Mann–Whitney *U* test for unpaired data. Graphpad Prism version 7.0 was used for statistical analyses and graphs (GraphPad, San Diego, CA).

3. Results

3.1. Design of the E2 tetramer for HCV-specific flow cytometry

The conformation of the rE2 product from the HCV Gt1a and Gt3a E2 plasmid transfectants was assessed pre- and post-biotinylation by ELISA, and revealed that the folding to maintain well-known epitopes was likely to be preserved (Supplementary Fig. 1).Two different forms of fluorescently-labelled biotinylated recombinant E2 (brE2) were prepared. The first was Gt1a or Gt3a brE2, which were tetramerized at different ratios to fluorescently-labelled streptavidin. The second form, which was ultimately used for detection of HCV E2-specific B cells, was a cocktail of Gt1a and Gt3a brE2 proteins tetramerized with fluorescently-labelled streptavidin.

3.2. Evaluation of Gt1a and Gt3a rE2 tetramers using HCV E2-specific hybridoma cells

An extensive optimization procedure to determine the best *w*/w ratio of tetramerized brE2 to streptavidin, and optimal fluorophores was performed using HCV-specific CBH-5 hybridoma cells, as the secreted monoclonal Abs from these cells bind both Gt1a and Gt3a rE2 in ELISA (Owsianka et al., 2008). As this hybridoma secretes IgG (rather than maintaining surface IgG expression) the cells were not expected to be uniformly positive, but all surface IgG positive hybridoma cells should be rE2 positive as the hybridoma cells are monoclonal.

First, the ratio of SA-PE to tetramerized Gt1a brE2 was tested at *w*/w ratios of: 1:0.5, 1:1, 1:2.5, 1:4 and 1:10. This initial testing was performed with a fixed staining amount of 15 μg of tetramer with 10^6 cells per tube for the Gt1a-specific tetramer. PE conjugated tetramers

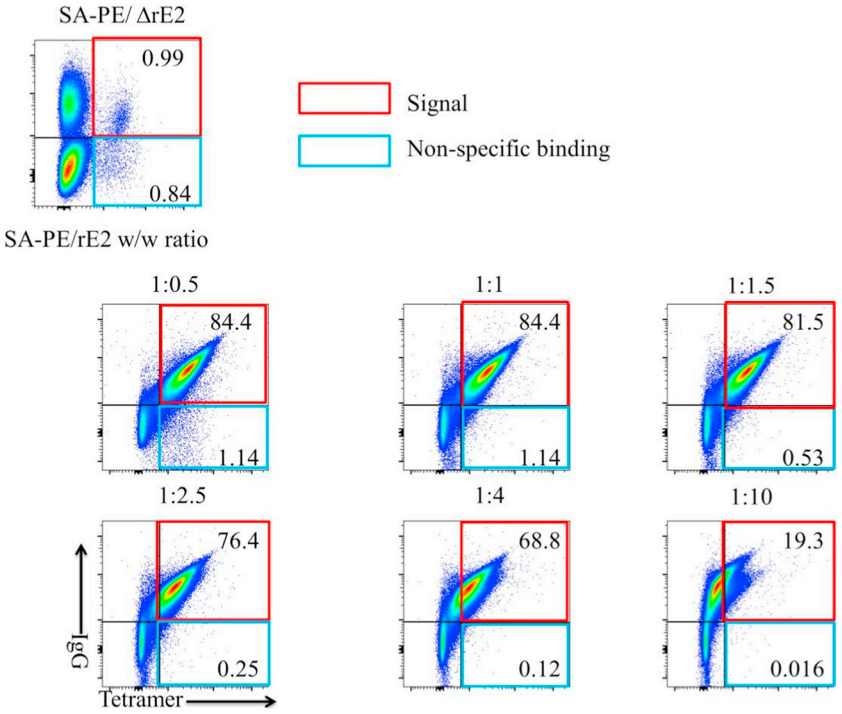

Fig. 1. Representative flow cytometry plots of Gt1a-rE2 tetramers developed using various ratios of SA-PE and brE2. CBH-5 cells which produce neutralizing antibodies against HCV E2 glycoprotein were stained with 15 μg tetramer constructed with various weight to weight (w/w) ratios of SA-PE and rE2. Tetramers prepared at different ratios were examined to evaluate the extent of specific binding ($IgG^+tetramer^+$; red box) and non-specific binding ($IgG^-tetramer^+$; blue box). rE2: recombinant E2. SA-PE: Streptavidin-Phycoerythrin.

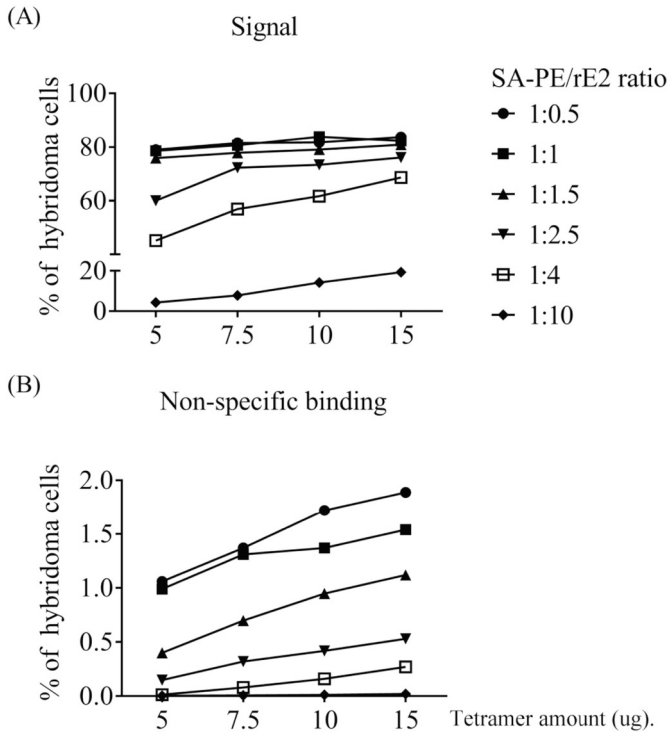

Fig. 2. Titration of tetramer against CBH-5. Various amounts of tetramer from 5 to 15 μg were used to stain 1×10^6 hybridoma cells to resolve the maximal signal (A) and minimum non-specific binding (B). SA-PE: Streptavidin-Phycoerythrin.

generated at all ratios resulted in a positive signal (frequency of $IgG^+tetramer^+$) between 19.3 and 84.4%, while still displaying a low level of non-specific binding (frequency of $IgG^-tetramer^+$) between 0.016 and 1.14% (Fig. 1). However, there was an expected trade-off evident where the lower ratio resulted in the lowest false negative (specificity), and the highest ratio had the lowest false positive (sensitivity). To explore the optimal staining conditions further, the Gt1a-specific tetramer was used to stain at different amounts (μg) of the tetramer in order to identify the optimal staining approach to obtain maximal signal, but with minimal non-specific binding. As shown in Fig. 2, CBH-5 cells were stained at various amounts (5, 7.5, 10 and 15 μg per million cells) along with various ratios of SA-PE to tetramerized Gt1a brE2. Increasing amounts of the tetramer probes improved the detection of signal, but also increased the non-specific binding. Interestingly, the Gt1a-specific tetramers composed of a high ratio of rE2 proteins inhibited positive detection, suggesting that brE2 unbound to PE may be competing with fluorescently-labelled tetramerized proteins for binding to CBH-5 cells. For all PBMCs spiked with CBH-5, a clear increase in signal was observed while increasing amounts of Gt1a-specific tetramers were used (Supplementary Fig. 2). Taken together, 15 μg of Gt1a-specific tetramer probe combined at 1:2.5 ratio of SA-PE to tetramerized Gt1a brE2 was selected for further staining.

For the Gt3a-specific tetramer, the ratio of various streptavidin dyes and brE2 were tested including 1:1, 1:2.5, 1:4 and 1:10 *w*/w ratio, in combination with four bright fluorophores: PE, APC, BV560 and BB515 with an initial staining concentration of 15 μg/1×10^6 cells. Similar to the results for the Gt1a-specific tetramer, PE conjugated Gt3a tetramers generated at a 1:1 and 1:2.5 ratio resulted in the maximal signal with CBH-5 cells (95.7% and 93.6%, respectively), while still displaying low levels of non-specific binding (0.99% and 0.17%, respectively). Staining with APC resulted in reasonably comparable levels to the PE, whereas significantly lower signal and higher non-specific binding were observed with BV650 and BB515 (Fig. 3). Also for BV650 and BB515, a large number of the IgG^+ cells failed to shift to the right in fluorescence intensity and be identified as $tetramer^+$, possibly due to these two fluorophores being less bright. As a result, only tetramers conjugated with either PE or APC were tested further.

3.3. Confirmation of E2 tetramer staining on PBMCs isolated from HCV infected subjects

To further investigate the sensitivity and specificity of the alternative fluorophores for Gt3a-specific tetramer staining, a preliminary evaluation of both the positive and background staining was tested in

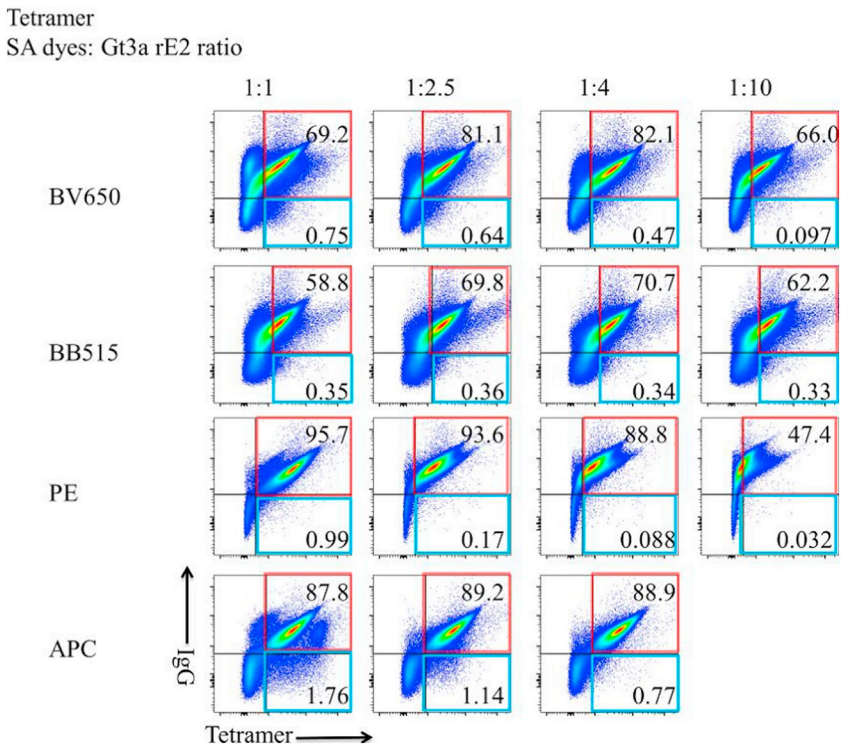

Fig. 3. Examination of various fluorophores for Gt3a-specific tetramer. After tetramerization with various SA dyes and Gt3a-brE2 at different ratios, CBH-5 cells were stained with 15 μg of the tetramers. SA-BB515 and BV650 gave the lowest true positive signal (red box) but with a high false negative signal (blue box). SA-PE and SA-APC gave the optimal signal at the 1:2.5 ratio.

two Gt3a-infected subjects and two uninfected healthy donors. For PE, the percent of tetramer$^+$ cells per million PBMCs in the two HCV-infected subjects were 0.58% and 0.62% of CD19$^+$CD20$^+$CD10$^-$IgD$^-$ (28 and 70 events per million cells, respectively), whereas the percentages of tetramer$^+$ cells in healthy donors (referred to as background) were 0.23% and 0.28% (10 and 17 events per million cells, respectively), giving a signal to background ratio of 3.6:1.0 (upper Fig. 4A). For the APC staining, a similar percentage of cells were tetramer$^+$ in the two HCV infected subjects, but the tetramer$^+$ percentage in the healthy donors was higher than for PE - resulting in a signal to background ratio of 1.5:1.0 for APC (lower Fig. 4A). Meanwhile, cells with PE labelling (tet-ΔrE2) elicited lower frequencies of naturally fluorophore-reactive B cells (0.016% to 0.061%) in comparison to that with APC labelling (0.72% to 0.98%) (Fig. 4B). Therefore, PE labelling of both Gt1a and Gt3a probes was selected for further studies.

3.4. Evaluation of Gt1a/Gt3a cocktail tetramers

Given that circulating HCV E2-specific B cells have been reported to be present at low frequency, the high background of APC as described above, was considered non-ideal. Nevertheless, as it is of considerable interest to be able to screen patient samples with brE2 tetramers representing multiple genotypes, the Gt1a- and Gt3a- brE2 were used to produce a cocktail Gt1a/3a PE tetramer incorporating both brE2s at a 1:2.5 ratio. The Gt1a and Gt3a brE2 proteins were mixed at a 2:1 (*w*/w) ratio, reflecting the optimal staining ratios of the individual tetramers (Supplementary Fig. 3). The staining efficiency of either Gt-specific or cocktail tetramers were compared using CBH-5 cells (Fig. 5). The cocktail tetramers were slightly more sensitive than individual Gt-specific tetramers (Gt1a: 93.6%, Gt3a: 94.4% versus Gt1a/3a: 96.0%) (Fig. 5A and B). Interestingly, the cocktail tetramer was associated with greatly reduced non-specific binding (tetramer$^+$/IgG$^-$) in comparison to Gt-specific tetramers (0.03% versus 0.84% or 0.20%, respectively for Gt1a and Gt3a tetramers) (Fig. 5A and B). Slightly lower background was also observed for two HCV-naïve human PBMCs when they were stained with either the cocktail tetramers or tetramer mixtures (a mix of Gt1a-specific and Gt3a-specific tetramers at a 2:1 ratios; Supplementary Fig. 3B). The reduction may be a result of loss of binding by B cells with limited cross-reactivity, and those whose BCRs need to crosslink to increase their binding avidity.

Subsequently, examination of the optimal cocktail tetramer staining was performed on PBMCs from healthy donors with various amounts (5, 7.5, and 10 μg for maximal 10×10^6 PBMCs) (Fig. 5C). Similarly, it was observed that an increase in background from healthy donors (CD19$^+$CD27$^+$IgG$^+$tetramer$^+$) coincided with increasing tetramer amounts (Fig. 5C). Hence, subsequent staining of human PBMCs was undertaken using 7.5 μg of cocktail tetramers. Finally, the efficacy of tetramer staining under three incubation temperatures was investigated: 4 °C, room temperature and 37 °C. The maximal signal from HCV-infected individuals and minimal background from healthy donors were detected with the 4 °C incubation (Fig. 6), likely to result from endocytosis of the antigen:BCR complex which should not occur at the lower temperature (Blery et al., 2006).

3.5. Validation of the specificity of E2 tetramer-positive sorted B cells by monoclonal Ab production

In order to further validate the specificity of the Gt1a/3a cocktail tetramer probe in detection of HCV E2 specific B cells, fluorescence activated cell sorting was used to single cell sort tetramer$^+$ B cells from a total of 9 samples from 4 subjects infected with either Gt1, Gt3, or both genotypes (Table 2). A total of 371 single B cells were sorted, before single cell RNA sequencing was completed, and the V_H and V_L transcripts identified. These sequences were natively paired and then co-expressed with a human IgG1 constant region in HEK293T cells. Of the 367 recombinant antibodies expressed, 321 (87.5%, range 67% to 100% for individual subjects) bound to either Gt1a or Gt3a rE2 by ELISA. Only approximately 25% of the antibodies bound both Gt1a and Gt3a rE2, but many of the antibodies were specific for one or other rE2 genotype (Fig. 7). Index tracing of the E2 fluorescence intensity of the sorted B cells did not associate with the mAb reactivity to E2 (Supplementary Fig. 4).

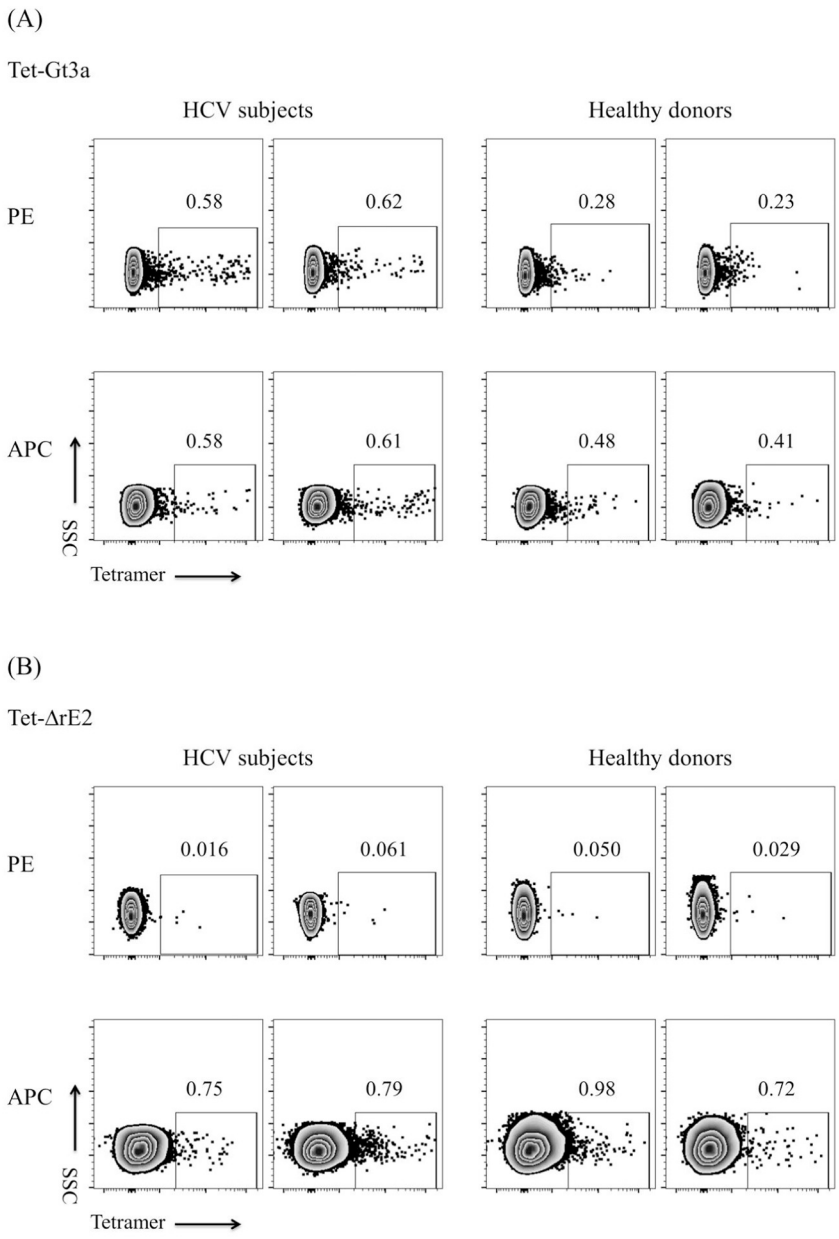

Fig. 4. Comparison of tetramers conjugated with either PE or APC. Tetramers conjugated with two different fluorophores, PE and APC labels were validated in human PBMCs from two HCV-infected and healthy donors. 15 μg of tetramers composed of Gt3a-rE2 (A) or ΔrE2 (B) were performed in individual tubes, revealing PE and APC. The percentage of HCV E2-specific B cells was defined as $CD19^+CD20^+CD10^-IgD^-tet^+$. PE: Phycoerythrin, APC: Allophycocyanin.

3.6. Successful detection of HCV E2-specific B cells in natural infection

To assess the utility of the optimized HCV Gt1a/3a cocktail tetramer probe for detection and characterization of HCV rE2-specific B cells during natural infection, human PBMC samples from HCV subjects and healthy donors were stained. The threshold for positivity was 0.049%, which was the mean frequency plus two standard deviations (SD) of HCV E2-specific B cells ($CD19^+CD20^+CD10^-IgD^-$ $tetramer^+$) from 10 healthy donors (Fig. 8).

A total of 33 HCV-infected individuals who had spontaneously cleared previous infection ($n = 17$; clearers), or were chronically infected ($n = 16$; chronics) with a known genotype were used for validation of the Gt1a/3a cocktail tetramer. HCV E2-specific B cells detected at a threshold above the designated threshold (0.049%) were identified in 29 of 33 HCV infected subjects (87.8%), including 15 clearers and 14 chronically infected subjects (Fig. 9). Plasma from the subjects without detectable $tetramer^+$ cells were subsequently screened for anti-E2 antibodies by ELISA, and all were E2 antibody negative (data not shown).

4. Discussion

This paper describes an optimized staining method for detection of HCV E2-specific B cells by flow cytometry. A careful consideration of tetramer amounts, *w*/w ratios, temperature and fluorophore for HCV E2-specific tetramers was performed to develop a highly sensitive assay that maintained a high specificity - an important issue given the low frequency of E2-specific B cells. Cocktail tetramers containing Gt1a and Gt3a brE2 was also described, increasing the sensitivity of the method. Both anti-E2 antibody hybridoma cell line (CBH-5) and cryopreserved PBMCs from HCV-infected subjects and healthy donors were used to develop the assay. The E2 cocktail tetramer staining was also applied to samples from subjects who had previously naturally cleared infection or developed chronic infection, with the great majority having HCV-rE2

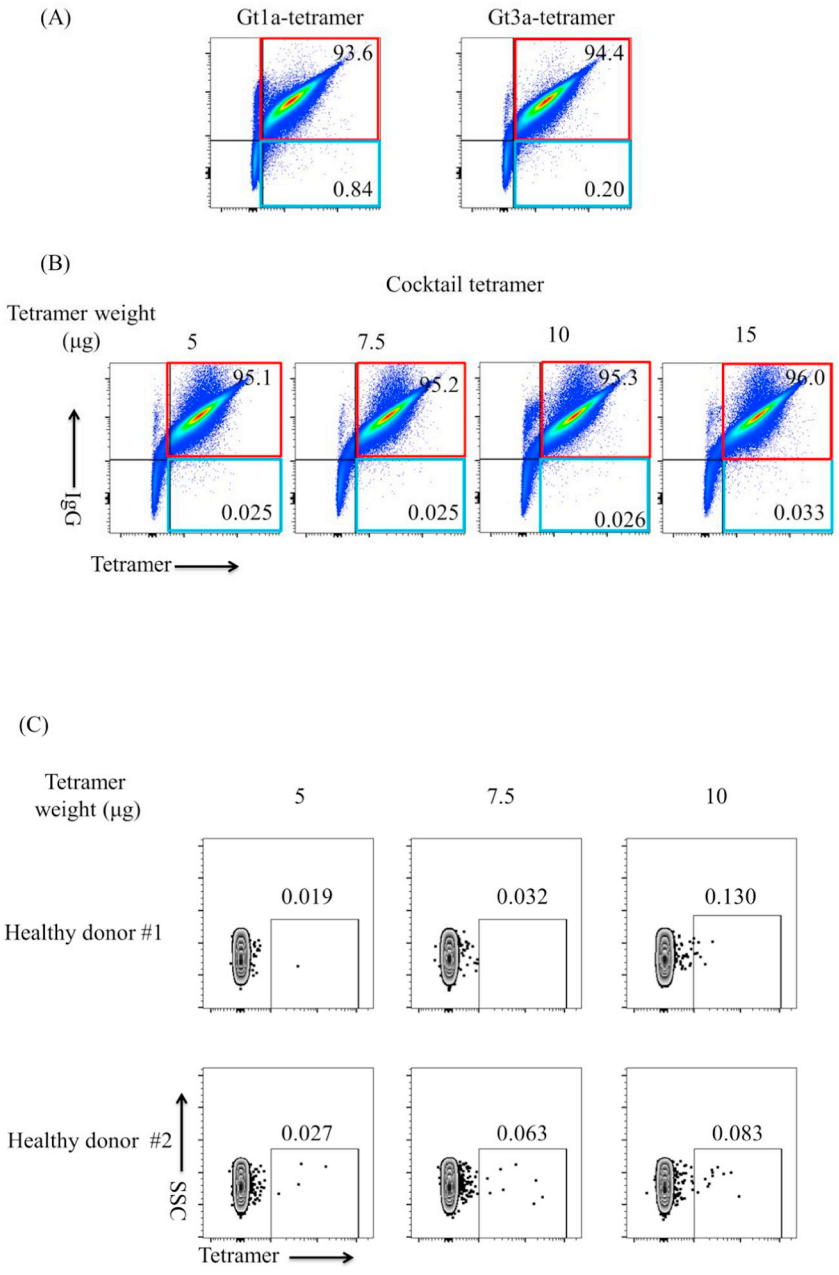

Fig. 5. Evaluation of a Gt1a/3a cocktail tetramer using CBH-5 cells and healthy PBMCs. (A) The efficacy of each Gt-specific tetramer (15 μg), Gt1a and Gt3a, were compared against CBH-5 hybridoma cells and comparable sensitivity was observed. (B and C). First, the Gt1a/Gt3a E2 cocktail (at a 2:1 *w*/w ratio) was tetramerized at a 1:2.5 ratio with SA-PE. The efficacy of the cocktail tetramers at various staining amounts (5, 7.5, 10 and 15 μg) was validated using CBH-5 (B) and PBMCs from healthy donors (C). The cocktail tetramer showed comparable detection and non-specific binding for the different amounts of cocktail tetramer used (B). However, there was an increased frequency of background ($CD19^+CD27^+IgD^-IgG^+tetramer^+$) in PBMCs from heathy donors when higher amounts of cocktail was used (C).

specific B cells detected at a low but comparable frequency in the clearers and chronics.

Qualitative assessments of antigen-specific memory B cells ex vivo has been challenging due to the low frequency of these cells in peripheral blood (Amanna and Slifka, 2006). Thus, both a sensitive and specific approach based on fluorescence labelling represents a major advance to allow visualization and characterization of low frequency B cell subsets. In principle, using a biotinylated antigenic protein conjugated to fluorescent dyes should provide an easy method for the detection of antigen-specific B cells via binding to the surface B cell receptor (Newman et al., 2003; Soldemo et al., 2014). The most successful probes developed to date for detection of antigen-specific B cells in HIV, dengue and influenza rely on the formation of multimer complexes, which are usually formed with a streptavidin-core and biotinylated glycoproteins (Scheid et al., 2009; Dosenovic et al., 2009), though background signal arising from B cells being naturally-reactive to the fluorophores is a potential limitation of this approach. Although tetramerization of T cell probes has been reported to minimize non-specific binding by the multimeric structure shielding fluorophores from non-specific binding to the fluorophores (Angelov et al., 2006), there appears to be evidence for this with one of our healthy subjects having higher binding to the delta E2 APC tetramer than to the E2 APC tetramer. PE- and APC-binding naïve B cells accounted for between 1:5000 and 1:25,000 of all mouse B cells, respectively after enrichment (Pape et al., 2011). As a result, several approaches have been widely implemented to exclude fluorophore-reactive B cells and increase the specificity of detecting antigen-specific B cells, such as decoy probes, tetramers conjugated with irrelative proteins and double-positive tetramers (Nanton et al., 2015; Rahe et al., 2018; Taylor et al., 2012; Brooks et al., 2018), which have proven particularly useful for identifying rare populations, such as naïve B cells (Brooks et al., 2018). In this study, higher specific staining was observed with PE, whereas higher frequencies of non-specific binding to the IgG^- hybridoma cells was observed with other fluorescent dyes. Given that HCV E2-specific B cells are present at low frequency it was regarded as essential that the optimized staining method had a low non-specific background signal to

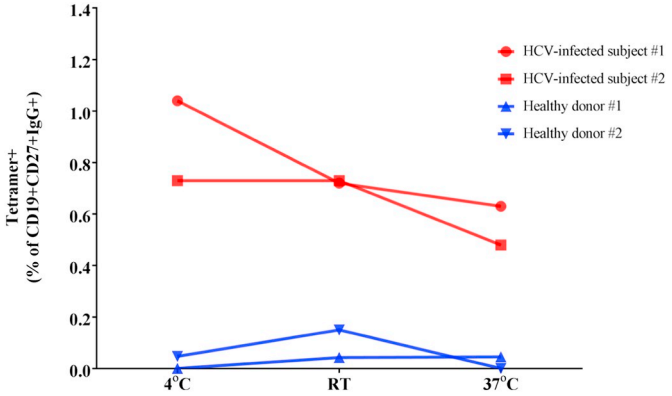

Fig. 6. Optimization of incubation temperature for the tetramer staining reagent. Cocktail tetramer staining was evaluated at three incubation temperatures for two independent HCV-infected participants and two healthy donors. HCV E2 specific class-switched memory B cells were gated on $CD19^+CD27^+IgG^+tetramer^+$. Staining at 4 °C was selected. RT: room temperature.

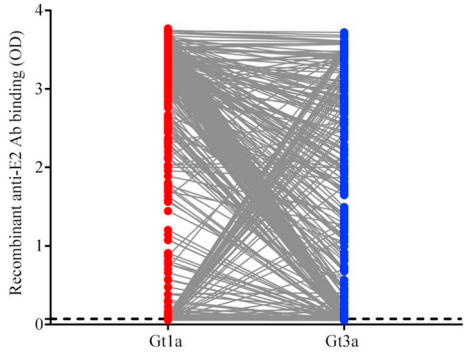

Fig. 7. Monoclonal antibody binding to either Gt1a or Gt3a E2. Binding titers of monoclonal Abs prepared from single cell sorted HCV E2-specific B cells identified using the Gt1a/3a cocktail tetramer. A total of 367 sorted HCV E2-specific single cells from 9 subjects were amplified, with 321 being reactive to E2 ELISA. The cut-off values were calculated as the mean of OD value plus three standard deviations (SD) from healthy plasma, which is 0.0711.

ensure specificity. Hence, only PE was considered suitable, and therefore the Gt1a and Gt3a brE2 proteins were combined in tetramers to allow the maximize sensitivity of detection of HCV E2-specific B cells It is worth noting that the cocktail tetramers appeared capable of increasing the avidity, and the frequency of BCRs with specificity for either Gt1a or Gt3a binding, without high background from HCV-naïve PBMCs compared with the tetramer mixture.

Analysis of the E2-specific B cell frequencies in the cross-sectional study reported here showed that the proportion of the combined HCV infection group with detectable $tetramer^+$ B cells was 29 of 33 subjects (87.8%), which is comparable to the single previous study which found 90% (28 out of 31) of subjects with chronic HCV infection to be positive (Boisvert et al., 2016). The four subjects without E2-specific B cells were also negative via ELISA using the same rE2, hence it remains unknown whether these samples were truly negative or the samples were not cross-reactive with the recombinant proteins used in this assay. It should be noted that the definition for positivity utilised here was not directly comparable to the previous report due to the flow cytometry configuration and surface markers included in the panel being slightly different ($CD19^+CD20^+CD10^-IgD^-$ versus $CD19^+CD27^+IgM^-$). Despite these minor differences, the sensitivity for detection of HCV-specific B cells was very similar between the two studies. The present study also detected similar frequencies of HCV-specific B cells between clearers and chronics (0.66 and 0.48%, respectively) and also similar frequencies to the clearers and chronics in the previous report (0.38 vs 0.47%, respectively) (Boisvert et al., 2016).

Monoclonal antibody production of the HCV E2-specific single sorted B cells suggested the two cocktail tetramers improved detection of HCV-specific B cells, because while approximately a quarter of the monoclonal antibodies generated from the B cells could bind both Gt1a and Gt3a, the majority could not. The monoclonal antibody production also indicated that the assay had good specificity as 87.5% of the cells stained by the tetramer encoded B cell receptors which showed specific-binding for HCV E2 (range 67 to 100% for individual subjects). This was consistent with a previous study in HIV, where the specificity of sorted anti-CD4 binding domain B cells was 87.1% (110 out of 127 mAbs) (Meffre et al., 2016). In this manuscript, we applied scRNAseq to identify the gene class usage for each BCR to allow in-Fusion cloning and mAb production, as scRNAseq is now widely being applied to characterise antigen-specific B cells. The advantages from this approach are that they allow you to obtain the RNA transcriptome data and pair it with BCR sequence and mAb phenotype. It should be mentioned that scRNAseq and pre-identification of the gene classes is not essential as a more economical and faster approach would be to pool the primers, as described previously (Liao et al., 2009).

In summary, this study describes the optimisation of a Gt1a- and Gt3a-E2 tetramers that can be used individually or in combination to identify HCV E2-specific B cells. This method combined with the previously described Gt2 E2 tetramer protocol (Boisvert et al., 2016), are important tools to allow sensitive high-throughput characterization of HCV E2-specific B cells. Despite over 30 years of research into HCV infection, the mechanisms leading to the chronic outcome from primary infection are incompletely defined. The development of Envelope-specific B cell tetramers has been instrumental in broadening both our understanding of HIV pathogenesis and also potential avenues for vaccine design. A similar impact is now feasible with these recent developments in HCV E2-specific B cell tools.

Table 2
Specificity of mAbs generated from Gt1a/3a tetramer sorted B cells.

Subject ID	Time point	Infecting	No. of BCRs	No. of Abs	No. reactive by ELISA to:		
	(DPI)	strain	amplified	made	E2 (%)	Gt1a E2	Gt3a E2
089	181	1b, 3a	46	46	41 (89)	40	27
089	754	1b, 3a	30	29	26 (90)	25	20
089	1155	3a	60	60	57 (95)	49	53
089	1741	3a	51	51	51 (100)	41	44
155	2250	3a	31	30	26 (87)	19	26
272	802	3a	47	47	42 (89)	34	26
272	2593	1a	47	45	30 (67)	29	19
087	132	1b	14	14	10 (71)	10	6
087	212	3a	45	45	38 (84)	37	25
Total no. (%)			371	367 (98.9)	321 (87.5)	284	246

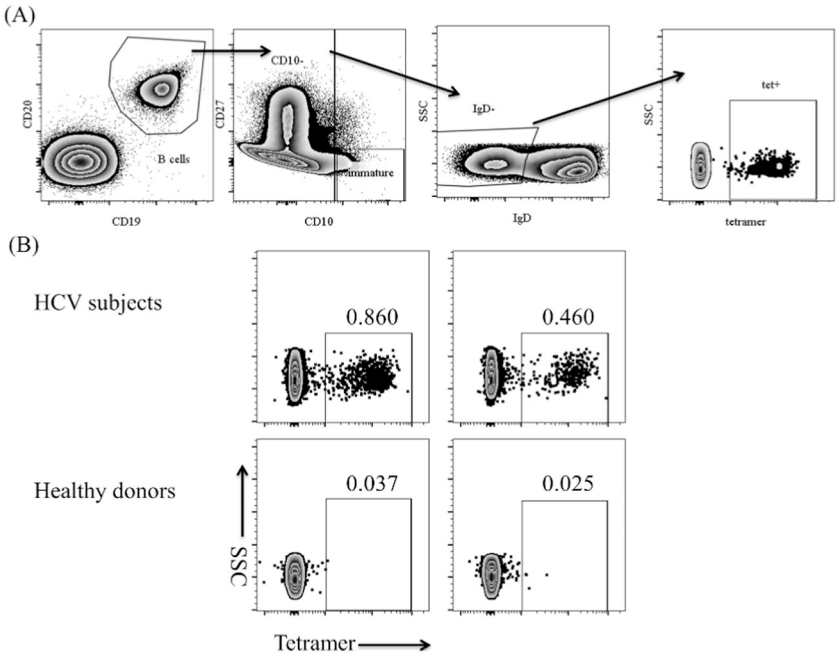

Fig. 8. Tetramer-based identification of HCV E2-specific B cells in two HCV-infected subjects and healthy donors using cocktail tetramers. (A) Representative flow plots for HCV-specific B cells. HCV E2-specific B cells were gated on $CD19^+CD20^+CD10^-IgD^-$tetramer$^+$. (B) HCV E2-specific B cells were validated on two independent HCV-infected individuals and healthy donors. Tet: tetramer.

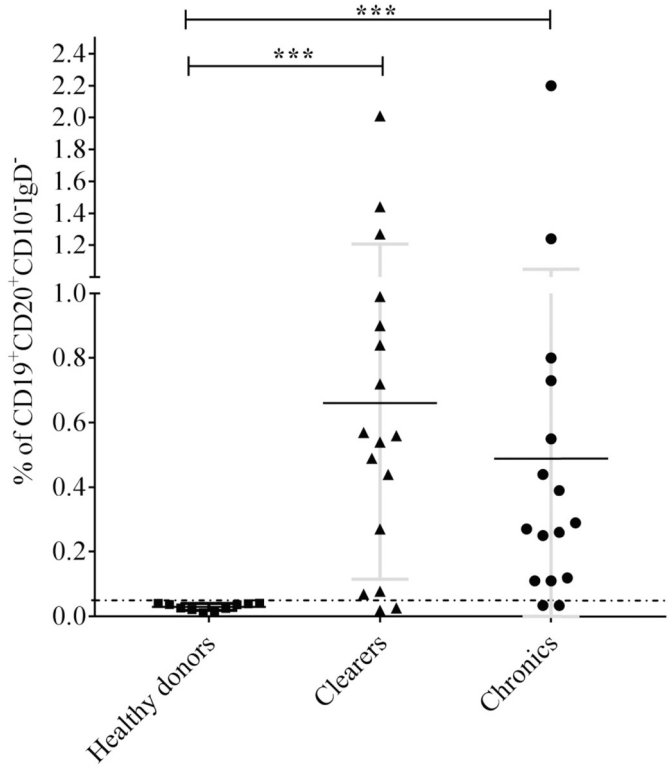

Fig. 9. HCV E2-specific B cell frequencies in ex vivo staining of PBMCs using cocktail tetramers on HCV-infected subjects who had previously cleared infection ($n = 17$) or had established chronic infection ($n = 16$). Each plot represents the frequency of HCV E2-specific B cells from the $CD19^+CD20^+CD10^-IgD^-$ B cell population. The threshold for reliable positive detection was determined using PBMCs from healthy blood donors ($n = 10$) and calculated as the mean frequency plus two standard deviations (SD) above the mean, which was 0.049%. Horizontal lines indicated the means, and error bars represented SD. Data were analyzed using the Mann–Whitney unpaired *t*-test. *** $p < .0001$.

Sources of funding

This work was supported by the Australian Government Department of Health National Health and Medical Research Council (NHMRC, http://www.nhmrc.gov.au) grants including NSW Health, Justice Health, and Corrective Services as partners (222887 and 1016351). AL and RB were supported by NHMRC Fellowships (1043067 and 1084706).

Disclosures

The authors have no financial conflicts of interest.

Acknowledgment

The HITS investigators include Kate Dolan, Paul Haber, William Rawlinson, Carla Treloar, Greg Dore, Lisa Maher, and Andrew Lloyd and Fabio Luciani. We also thanks for Chris Brownlee and Emma Johansson Beves in the Flow Facility of the Mark Wainwright Analytical Centre, University of New South Wales for helpful technical assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2019.06.016.

References

Alhammad, Y., Gu, J., Boo, I., Harrison, D., McCaffrey, K., Vietheer, P.T., Edwards, S., Quinn, C., Coulibaly, F., Poumbourios, P., Drummer, H.E., 2015. Monoclonal antibodies directed toward the hepatitis C virus glycoprotein E2 detect antigenic differences modulated by the N-terminal hypervariable region 1 (HVR1), HVR2, and intergenotypic variable region. J. Virol. 89, 12245–12261.

Amanna, I.J., Slifka, M.K., 2006. Quantitation of rare memory B cell populations by two independent and complementary approaches. J. Immunol. Methods 317, 175–185.

Angelov, G.S., Guillaume, P., Cebecauer, M., Bosshard, G., Dojcinovic, D., Baumgaertner, P., Luescher, I.F., 2006. Soluble MHC-peptide complexes containing long rigid linkers abolish CTL-mediated cytotoxicity. J. Immunol. 176, 3356–3365.

Bartosch, B., Dubuisson, J., Cosset, F.L., 2003. Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes. J. Exp. Med. 197, 633–642.

Blery, M., Tze, L., Miosge, L.A., Jun, J.E., Goodnow, C.C., 2006. Essential role of membrane cholesterol in accelerated BCR internalization and uncoupling from NF-kappa B in B cell clonal anergy. J. Exp. Med. 203, 1773–1783.

Boisvert, M., Zhang, W., Elrod, E.J., Bernard, N.F., Villeneuve, J.P., Bruneau, J., Marcotrigiano, J., Shoukry, N.H., Grakoui, A., 2016. Novel E2 glycoprotein tetramer detects hepatitis C virus-specific memory B cells. J. Immunol. 197, 4848–4858.
Borgia, S.M., Hedskog, C., Parhy, B., Hyland, R.H., Stamm, L.M., Brainard, D.M., Subramanian, M.G., McHutchison, J.G., Mo, H., Svarovskaia, E., Shafran, S.D., 2018. Identification of a novel hepatitis C virus genotype from Punjab, India: expanding classification of hepatitis C virus into 8 genotypes. J. Infect. Dis. 218, 1722–1729.
Brooks, J.F., Liu, X., Davies, J.M., Wells, J.W., Steptoe, R.J., 2018. Tetramer-based identification of naive antigen-specific B cells within a polyclonal repertoire. Eur. J. Immunol. 48, 1251–1254.
Carlsen, T.H., Scheel, T.K., Ramirez, S., Foung, S.K., Bukh, J., 2013. Characterization of hepatitis C virus recombinants with chimeric E1/E2 envelope proteins and identification of single amino acids in the E2 stem region important for entry. J. Virol. 87, 1385–1399.
Ciczora, Y., Callens, N., Penin, F., Pecheur, E.I., Dubuisson, J., 2007. Transmembrane domains of hepatitis C virus envelope glycoproteins: residues involved in E1E2 heterodimerization and involvement of these domains in virus entry. J. Virol. 81, 2372–2381.
Degauque, N., Elong Ngono, A., Akl, A., Lepetit, M., Crochette, R., Giral, M., Lepourry, J., Pallier, A., Castagnet, S., Dugast, E., Guillot-Gueguen, C., Jacq-Foucher, M., Saulquin, X., Cesbron, A., Laplaud, D., Nicot, A., Brouard, S., Soulillou, J.P., 2013. Characterization of antigen-specific B cells using nominal antigen-coated flow-beads. PLoS One 8, e84273.
Dolan, K., Teutsch, S., Scheuer, N., Levy, M., Rawlinson, W., Kaldor, J., Lloyd, A., Haber, P., 2010. Incidence and risk for acute hepatitis C infection during imprisonment in Australia. Eur. J. Epidemiol. 25, 143–148.
Dosenovic, P., Chakrabarti, B., Soldemo, M., Douagi, I., Forsell, M.N., Li, Y., Phogat, A., Paulie, S., Hoxie, J., Wyatt, R.T., Karlsson Hedestam, G.B., 2009. Selective expansion of HIV-1 envelope glycoprotein-specific B cell subsets recognizing distinct structural elements following immunization. J. Immunol. 183, 3373–3382.
Douam, F., Dao Thi, V.L., Maurin, G., Fresquet, J., Mompelat, D., Zeisel, M.B., Baumert, T.F., Cosset, F.L., Lavillette, D., 2014. Critical interaction between E1 and E2 glycoproteins determines binding and fusion properties of hepatitis C virus during cell entry. Hepatology 59, 776–788.
Facility, N.T.C., 2018. Class I MHC Tetramer Preparation protocol.
Falade-Nwulia, O., Sulkowski, M.S., Merkow, A., Latkin, C., Mehta, S.H., 2018. Understanding and addressing hepatitis C reinfection in the oral direct-acting antiviral era. J. Viral Hepat. 25, 220–227.
Flint, M., Dubuisson, J., Maidens, C., Harrop, R., Guile, G.R., Borrow, P., McKeating, J.A., 2000. Functional characterization of intracellular and secreted forms of a truncated hepatitis C virus E2 glycoprotein. J. Virol. 74, 702–709.
Hoshida, Y., Fuchs, B.C., Bardeesy, N., Baumert, T.F., Chung, R.T., 2014. Pathogenesis and prevention of hepatitis C virus-induced hepatocellular carcinoma. J. Hepatol. 61, S79–S90.
Khan, A.G., Whidby, J., Miller, M.T., Scarborough, H., Zatorski, A.V., Cygan, A., Price, A.A., Yost, S.A., Bohannon, C.D., Jacob, J., Grakoui, A., Marcotrigiano, J., 2014. Structure of the core ectodomain of the hepatitis C virus envelope glycoprotein 2. Nature 509, 381–384.
Liao, H.X., Levesque, M.C., Nagel, A., Dixon, A., Zhang, R., Walter, E., Parks, R., Whitesides, J., Marshall, D.J., Hwang, K.K., Yang, Y., Chen, X., Gao, F., Munshaw, S., Kepler, T.B., Denny, T., Moody, M.A., Haynes, B.F., 2009. High-throughput isolation of immunoglobulin genes from single human B cells and expression as monoclonal antibodies. J. Virol. Methods 158, 171–179.
Lindenbach, B.D., Evans, M.J., Syder, A.J., Wolk, B., Tellinghuisen, T.L., Liu, C.C., Maruyama, T., Hynes, R.O., Burton, D.R., McKeating, J.A., Rice, C.M., 2005. Complete replication of hepatitis C virus in cell culture. Science 309, 623–626.
Luciani, F., Bretana, N.A., Teutsch, S., Amin, J., Topp, L., Dore, G.J., Maher, L., Dolan, K., Lloyd, A.R., HI-p, investigators, 2014. A prospective study of hepatitis C incidence in Australian prisoners. Addiction 109, 1695–1706.
Maurin, G., Fresquet, J., Granio, O., Wychowski, C., Cosset, F.L., Lavillette, D., 2011. Identification of interactions in the E1E2 heterodimer of hepatitis C virus important for cell entry. J. Biol. Chem. 286, 23865–23876.
McCaffrey, K., Boo, I., Owczarek, C.M., Hardy, M.P., Perugini, M.A., Fabri, L., Scotney, P., Poumbourios, P., Drummer, H.E., 2017. An Optimized Hepatitis C Virus E2 Glycoprotein Core Adopts a Functional Homodimer That Efficiently Blocks Virus Entry. J. Virol. 91.
Meffre, E., Louie, A., Bannock, J., Kim, L.J.Y., Ho, J., Frear, C.C., Kardava, L., Wang, W., Buckner, C.M., Wang, Y., Fankuchen, O.R., Gittens, K.R., Chun, T.-W., Li, Y., Fauci, A.S., Moir, S., 2016. Maturational characteristics of HIV-specific antibodies in viremic individuals. JCI insight 1, e84610.
Moody, M.A., Haynes, B.F., 2008. Antigen-specific B cell detection reagents: use and quality control. Cytometry A 73, 1086–1092.
Nanton, M.R., Lee, S.J., Atif, S.M., Nuccio, S.P., Taylor, J.J., Baumler, A.J., Way, S.S., McSorley, S.J., 2015. Direct visualization of endogenous Salmonella-specific B cells reveals a marked delay in clonal expansion and germinal center development. Eur. J. Immunol. 45, 428–441.
Newman, J., Rice, J.S., Wang, C., Harris, S.L., Diamond, B., 2003. Identification of an antigen-specific B cell population. J. Immunol. Methods 272, 177–187.
Owsianka, A.M., Tarr, A.W., Keck, Z.Y., Li, T.K., Witteveldt, J., Adair, R., Foung, S.K., Ball, J.K., Patel, A.H., 2008. Broadly neutralizing human monoclonal antibodies to the hepatitis C virus E2 glycoprotein. J. Gen. Virol. 89, 653–659.
Pape, K.A., Taylor, J.J., Maul, R.W., Gearhart, P.J., Jenkins, M.K., 2011. Different B cell populations mediate early and late memory during an endogenous immune response. Science 331, 1203–1207.
Petruzziello, A., Marigliano, S., Loquercio, G., Cozzolino, A., Cacciapuoti, C., 2016. Global epidemiology of hepatitis C virus infection: An up-date of the distribution and circulation of hepatitis C virus genotypes. World J. Gastroenterol. 22, 7824–7840.
Polaris Observatory, H.C.V.C., 2017. Global prevalence and genotype distribution of hepatitis C virus infection in 2015: a modelling study. Lancet Gastroenterol. Hepatol. 2, 161–176.
Rahe, M.C., Gustafson, K.L., Murtaugh, M.P., 2018. B cell tetramer development for veterinary vaccinology. Viral Immunol. 31, 1–10.
Rizzetto, S., Koppstein, D.N.P., Samir, J., Singh, M., Reed, J.H., Cai, C.H., Lloyd, A.R., Eltahla, A.A., Goodnow, C.C., Luciani, F., 2018. B-cell receptor reconstruction from single-cell RNA-seq with VDJPuzzle. Bioinformatics 34, 2846–2847.
Scheid, J.F., Mouquet, H., Feldhahn, N., Walker, B.D., Pereyra, F., Cutrell, E., Seaman, M.S., Mascola, J.R., Wyatt, R.T., Wardemann, H., Nussenzweig, M.C., 2009. A method for identification of HIV gp140 binding memory B cells in human blood. J. Immunol. Methods 343, 65–67.
Scott, N., McBryde, E., Vickerman, P., Martin, N.K., Stone, J., Drummer, H., Hellard, M., 2015. The role of a hepatitis C virus vaccine: modelling the benefits alongside direct-acting antiviral treatments. BMC Med. 13, 198.
Shoukry, N.H., 2018. Hepatitis C vaccines, antibodies, and T Cells. Front. Immunol. 9, 1480.
Simmonds, P., Bukh, J., Combet, C., Deleage, G., Enomoto, N., Feinstone, S., Halfon, P., Inchauspe, G., Kuiken, C., Maertens, G., Mizokami, M., Murphy, D.G., Okamoto, H., Pawlotsky, J.M., Penin, F., Sablon, E., Shin, I.T., Stuyver, L.J., Thiel, H.J., Viazov, S., Weiner, A.J., Widell, A., 2005. Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. Hepatology 42, 962–973.
Soldemo, M., Pedersen, G.K., Karlsson Hedestam, G.B., 2014. HIV-1 Env-specific memory and germinal center B cells in C57BL/6 mice. Viruses 6, 3400–3414.
Sundling, C., Li, Y., Huynh, N., Poulsen, C., Wilson, R., O'Dell, S., Feng, Y., Mascola, J.R., Wyatt, R.T., Karlsson Hedestam, G.B., 2012. High-resolution definition of vaccine-elicited B cell responses against the HIV primary receptor binding site. Sci. Transl. Med. 4, 142ra96.
Tarr, A.W., Owsianka, A.M., Szwejk, A., Ball, J.K., Patel, A.H., 2007. Cloning, expression, and functional analysis of patient-derived hepatitis C virus glycoproteins. Methods Mol. Biol. 379, 177–197.
Taylor, J.J., Martinez, R.J., Titcombe, P.J., Barsness, L.O., Thomas, S.R., Zhang, N., Katzman, S.D., Jenkins, M.K., Mueller, D.L., 2012. Deletion and anergy of polyclonal B cells specific for ubiquitous membrane-bound self-antigen. J. Exp. Med. 209, 2065–2077.
Teutsch, S., Luciani, F., Scheuer, N., McCredie, L., Hosseiny, P., Rawlinson, W., Kaldor, J., Dore, G.J., Dolan, K., Ffrench, R., Lloyd, A., Haber, P., Levy, M., 2010. Incidence of primary hepatitis C infection and risk factors for transmission in an Australian prisoner cohort. BMC Public Health 10, 633.
Umemura, T., Wang, R.Y., Schechterly, C., Shih, J.W., Kiyosawa, K., Alter, H.J., 2006. Quantitative analysis of anti-hepatitis C virus antibody-secreting B cells in patients with chronic hepatitis C. Hepatology 43, 91–99.
Underwood, A.P., Walker, M.R., Brasher, N.A., Eltahla, A.A., Maher, L., Luciani, F., Lloyd, A.R., Bull, R.A., 2018. Understanding the Determinants of BnAb Induction in Acute HCV Infection. Viruses 10, 659.
Walker, M.R., Li, H., Teutsch, S., Betz-Stablein, B., Luciani, F., Lloyd, A.R., Bull, R.A., 2016. Incident Hepatitis C Virus Genotype Distribution and Multiple Infection in Australian Prisons. J. Clin. Microbiol. 54, 1855–1861.
Wang, Y., Keck, Z.Y., Saha, A., Xia, J., Conrad, F., Lou, J., Eckart, M., Marks, J.D., Foung, S.K., 2011. Affinity maturation to improve human monoclonal antibody neutralization potency and breadth against hepatitis C virus. J. Biol. Chem. 286, 44218–44233.