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Development and characterization of a human monoclonal antibody targeting the N-terminal region of hepatitis C virus envelope glycoprotein E1

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Abstract

Monoclonal antibodies (mAbs) targeting the hepatitis C virus (HCV) envelope have been raised mainly against envelope protein 2 (E2), while the antigenic epitopes of envelope protein 1 (E1) are not fully identified. Here we describe the detailed characterization of a human mAb, designated A6, generated from an HCV genotype 1b infected patient. ELISA results showed reactivity of mAb A6 to full-length HCV E1E2 of genotypes 1a, 1b and 2a. Epitope mapping identified a region spanning amino acids 230-239 within the N-terminal region of E1 as critical for binding. Antibody binding to this epitope was not conformation dependent. Neutralization assays showed that mAb A6 lacks neutralizing capacity and does not interfere with the activity of known neutralizing antibodies. In summary, mAb A6 is an important tool to study the structure and function of E1 within the viral envelope, a crucial step in the development of an effective prophylactic HCV vaccine.

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1. Introduction

Hepatitis C virus (HCV) is a member of the *Hepacivirus* genus within the *Flaviviridae* family. Based on the 9.6kb-long RNA genome sequence, HCV is classified into 7 genotypes (1-7) and multiple subtypes (a, b, c, etc.) (Bukh, 2016). More than 170 million people worldwide are estimated to be infected with HCV (Petruzziello et al., 2016). More than 70% of individuals with acute HCV infection will become chronically infected, which after several decades may lead to liver cirrhosis and hepatocellular carcinoma. Despite the remarkable improvement of HCV treatment regimens using direct-acting antivirals (DAAs), mutations occur that bring about resistance and negatively impact treatment outcome (Li and De Clercq, 2017; Pawlotsky, 2016). The risk of acquiring HCV infection is high among injection drug users due to the high prevalence of HCV in this population. The high mutation rate of HCV, leading to continuous release of closely related viral variants that escape the host's adaptive immune response, represents a major hurdle for the development of a vaccine. Identification of conserved epitopes that induce protective immunity would strongly promote vaccine design.

Inside the host cell, viral RNA is translated into a single polyprotein precursor that is cleaved into structural (core, envelope E1 and E2) and non-structural (P7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) proteins (Bartenschlager et al., 2011). Structural proteins are responsible for the formation of the HCV virus particle, while the non-structural proteins are essential for viral replication, translation and assembly. Our insight in the HCV life cycle was hampered for a long time but the development of the HCV pseudoparticle (HCVpp) and HCV cell culture (HCVcc) systems paved the way for a more comprehensive exploration of virus infectivity (Hsu et al., 2003; Wakita et al., 2005). Both systems are now widely used for studying HCV entry and screening for viral inhibitors (Catanese and Dorner, 2015). The envelope proteins E1 and E2 are highly glycosylated membrane-associated proteins consisting of an N-terminal ectodomain and a C-terminal transmembrane domain. The E1 protein is 192 amino acids (AA) long (from AA 192 to 383) while the E2 glycoprotein is 363 residues (AA 384 to 746) in length according to the reference strain H77 (accession no. AF011751). E2 functions as mediator for viral entry through interaction with host attachment factors and receptors (Dubuisson and Cosset, 2014). It is also the main target for the host's adaptive immune system. Recently the E1 protein was also shown to be involved in viral entry and virion assembly (Haddad et al., 2017).

The essential role of cell-mediated immunity in HCV clearance has been extensively reported (Abdelwahab, 2016). The early generation of neutralizing antibodies has been associated with resistance to infection in individuals at high-risk of exposure, spontaneous clearance during acute infection and sustained virologic response after therapy (Ndongo et al., 2010; Osburn et al., 2014; Swann et al., 2016). Although the envelope of HCV contains multiple immunogenic epitopes, the majority of monoclonal antibodies (mAbs) isolated from infected patients or vaccinated animals have been identified as E2-specific (Tabll et al., 2015). The biological activity of these antibodies is diverse and varies from neutralizing to non-neutralizing or even interfering (Wang et al., 2011a). Polyclonal antibodies from HCV-infected patients were able to protect animal models like humanized mice and chimpanzees from HCV challenge (Bukh et al., 2015; Meuleman et al., 2011; Vanwolleghem et al., 2008). We and others previously reported that neutralizing antibodies targeting the E2 protein could protect from HCV infection *in vitro* and *in vivo* (Desombere et al., 2016; Keck et al., 2016; Mesalam et al., 2016). In addition, administration of mAb MBL-HCV1, targeting E2, delayed viral rebound following liver transplantation, while complete protection was reported when combined with the polymerase inhibitor sofosbuvir (Chung et al., 2013; Smith et al., 2017). These observations point towards the importance of humoral immunity and neutralizing antibodies in HCV clearance.

Despite the availability of multiple *in vitro* systems as well as experimental animal models, little is still known about the structure and actual function of the E1 glycoprotein (Douam et al., 2014; Haddad et al., 2017; Lavillette et al., 2007; Wahid et al., 2013). Evidence for the immunogenicity and the induction of neutralizing antibodies by E1 has been reported. However, only few mAbs have been raised against this protein compared to the numerous anti-E2 mAbs described in the literature. This may be related to the difficulty to express the E1 protein as a correctly folded monomer (Op De Beeck et al., 2001). Polyclonal antibodies from mice vaccinated with E1-HCVpp or recombinant E1 protein were able to neutralize HCVcc (Dreux et al., 2006; Pietschmann et al., 2006). Also, synthetic peptides covering the C terminal region of E1 were able to react with 32% of sera from infected patients in one study (Siemoneit et al., 1995) while 92% reactivity was reported in another study (Ray et al., 1994). In addition, immune sera of rabbits vaccinated with a synthetic peptide encompassing AA 315-323 prevented the binding and entry of HCV particles into HepG2 cells (El-Awady et al., 2006). However, this finding has not been confirmed using the HCVpp or HCVcc systems.

Two main regions have been identified as reactive domains for anti-E1 antibodies. The first region is the N- terminal region identified by the human mAb H-111 (AA 192-202) (Keck et al., 2004b) and the murine mAb A4 (AA 197-207) (Dubuisson et al., 1994). While these antibodies recognize E1 presented on the viral envelope, mAb H-111 is only weakly neutralizing while no neutralization has been reported for mAb A4. The second region encompasses AA 313-327, which is located at the C terminus and is identified mainly by mAbs IGH505 and IGH526 (Kong et al., 2015; Meunier et al., 2008). We isolated a monoclonal antibody, designated A6, from a HCV genotype 1b infected patient. Epitope mapping identified AA 230-239 within the N-terminal region of E1 as the critical site for binding. This mAb showed affinity towards a panel of HCV envelope proteins of genotypes 1a, 1b and 2a, but lacked any neutralizing or interfering activity.

2. Materials and Methods

2.1. *Human sera, cell lines and antibodies*

Blood samples were collected from HCV infected patients who were followed at the Ghent University Hospital. The study was approved by the local ethical committee and all patients involved gave informed consent. Human embryonic kidney cells (293T) and human hepatoma cell lines (Huh-7.5RFP-NLS-IPS and Hep3B) were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum, 1% non-essential amino acids, 1% L-glutamine and antibiotics. The human anti-E1 mAb IGH526, the murine anti-E1 mAb A4, the murine anti-E2 mAb AP33 and the human anti-E2 mAbs MRCT10, 1:7, HC84.26, CBH-7 and HC-1AM were previously described (Allander et al., 2000; Dubuisson et al., 1994; Keck et al., 2004a; Keck et al., 2012; Kong et al., 2015; Owsianka et al., 2001; Pantua et al., 2013; Wang et al., 2011b).

2.2. *Amplification and expression of HCV envelope*

RNA was extracted from the plasma of infected patients using ZR Viral RNA kit (Zymo Research) followed by cDNA synthesis using superscript III reverse transcriptase (Invitrogen) and random primers. The full-length E1E2 sequence was amplified by nested PCR. The first PCR was performed using LongAmp DNA polymerase (NEB) and the following primers: (F) 5`-CGT AGG TCG CGT AAC TTG GGT AA-3′ and (R) 5`-GTG CGC CTC GGC CCT GGT GAT AAA-3`. The second-round PCR was performed using Pfu DNA polymerase (Promega) and primers: (F) 5'-TAT AGA TAT CAT GGG GTA CAT TCC GCT CGT C-3' and (R) 5'-ATA TGA TAT CTT ACT CAG CCT GAG CTA TCA G-3'. PCR products were analyzed using 1% agarose gel electrophoresis and the bands corresponding to E1E2 were eluted and cloned into the pCDNA3.1/Hygro expression vector (Invitrogen). The inserts were sequenced (Center for Medical Genetics, Ghent University, Belgium), multiple aligned and analyzed using BioEdit version 7.2.0, Clone manager 9 professional and CLC main workbench version 7.6.4 (QIAGEN). HCV E1E2 sequences covering all 7 genotypes (1a, 1b, 2a, 2b, 3a, 4a, 5a, 6a and 7a) were retrieved from the European HCV database (https://euhcvdb.ibcp.fr/euHCVdb/) and the NCBI (https://www.ncbi.nlm.nih.gov/nucleotide/) and used for alignment with Belgian isolates. Based on amino acid sequences of the whole E1E2, neighbor joining phylogenetic tree was constructed using 1000 replicates bootstrapping analysis of CLC main workbench version 7.6.4. For expression of E1E2 in mammalian cells, constructs encoding the E1E2 region of genotypes 1-6 were used for transfection of 293T cells using ProFection mammalian transfection kit (Promega). After 48 hours, lysis buffer (Promega) and protease inhibitors (Roche) were added and the cell lysate was centrifuged at 13,000 rpm and 4°C. The supernatant was collected and stored at –80°C until use. Constructs containing the following viral strains were used: H77, UKN1A20.8, UKN1A14.38, J4, UKN1B5.23, UKN1B12.16, P5VD, P5VE, P5VF, JFH1, UKN2A1.2, UKN2B2.8, S52, UKN4.11.1, UKN5.14.4 and UKN6.5.8 in addition to 28 Belgian isolates (Desombere et al., 2016; Fafi-Kremer et al., 2010; Lavillette et al., 2005; Owsianka et al., 2005).

2.3. Generation of anti-HCV mAb

The hybridoma cells were generated from the peripheral blood mononuclear cells (PBMCs) of a genotype 1b HCV infected patient as described previously (Depraetere et al., 2001). Clones that secreted E1-specific antibodies were identified using a prototype version of the INNO-LIA® HCV antibody test (Innogenetics, Belgium). After subcloning, one E1-specific hybridoma cell line was retained and designated A6. Cells were propagated in a specific antibody production bioreactor (Integra) and the culture supernatant was harvested. mAb A6 was purified using a protein G column (GE Healthcare Life Sciences), concentrated using Amicon centrifugal filters with 50 kDa cut-off (Merck Millipore) and the concentration was estimated using a commercial human IgG ELISA quantification kit according to manufacturer instructions (Bethyl Laboratories).

2.4. GNA binding, competition and denaturation ELISA

The binding affinity of mAb A6 to cell lysate containing recombinant HCV envelope glycoproteins was tested using GNA binding ELISA as previously described with some modifications (Owsianka et al., 2005). Briefly, ELISA plates were coated with Galanthus nivalis lectin (GNA) and incubated overnight at 4°C. The next day, plates were washed (PBS-0.05% Tween 20) and blocked (PBS-5% BSA) for 1 hour at room temperature (RT), after which cell lysate was added and incubated for 2 hours. After washing, diluted mAbs (PBS-5% BSA, 20% goat serum, 0.05% Tween 20) were added and plates were incubated

1.5 hours followed by addition of HRP-conjugated goat anti-host IgG (anti-human for A6 and 1:7 or anti-mouse for A4). In case of peptide competition assay, serial dilutions of peptides were mixed with a fixed concentration of mAb A6 (20 µg/mL) before addition to wells. TMB substrate was added and the optical density (OD) was read at 450nm. Competition ELISA was performed using a previously described protocol with some modifications (Potter et al., 2012). Briefly, the GNA-coated plates were loaded with E1E2 cell lysate diluted in blocking buffer (2.5% BSA, 2.5% goat serum, 0.1% Tween 20) and incubated for 2 hours. Plates were washed and serial dilutions of competing mAbs (A6 or MRCT10) were added. After 1 hour, biotinylated AP33 was added at a concentration corresponding to 60-75% of the maximum OD value and incubated for 1 hour. Plates were washed and HRP-conjugated streptavidin was added for 30 min. In denaturation ELISA, cell lysates were incubated with 0.5% sodium dodecyl sulfate (SDS) and 5 mM dithiothreitol (DTT) for 15 min at 56°C (Keck et al., 2012).

2.5. *Protein electrophoresis and Immunoblotting*

To test the specificity of mAb A6 binding, lysate of 293T cells expressing H77 E1E2 was used in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Samples were mixed with 4X Bolt LDS sample and reducing buffers (Life Technologies) followed by heating at 70°C for 10 minutes. In case of deglycosylation, the cell lysate was first treated with PNGase F or Endo H (NEB) according to manufacturer instructions. Samples were then resolved on 12% acrylamide gel followed by transfer to nitrocellulose membrane and blocked for one hour at RT using 5% non-fat milk in TBS-T (TBS-0.05% Tween 20). After washing (TBS-T), mAb A6 was added (1 µg/mL) followed by 2 hours incubation at RT. HRP-conjugated goat anti-human IgG (Fc-specific) was added followed by one hour incubation. Bands were visualized using Pierce ECL Plus western blotting substrate (Thermo Scientific) and imaged on an ImageQuant LAS 4000 (GE Healthcare Life Sciences). Molecular weights were estimated based on the MagicMark XP western protein standard (Invitrogen).

2.6. *Epitope mapping*

To identify the region critical for mAb binding, a 15-mer, 14-AA overlapping peptide microarray (PEPperCHIP) was used according to the manufacturer's instructions (PEPperPRINT GmbH, Germany).

The peptides printed on the chip were designed to cover the entire E1E2 sequence of a HCV genotype 1b isolate. The sequences and the layout of peptides spotted on the chip are presented in Supplementary File 1. For peptide ELISA, plates were loaded with 2.5 µg/well of dissolved peptides (GenScript, USA) and incubated overnight at 4°C. After washing and blocking for 1 hour, serial dilutions of mAb A6 were added followed by incubation for 1 hour. Plates were washed and HRP-conjugated goat-anti-human IgG was added. After 1 hour of incubation, plates were washed, TMB substrate was added and the OD was measured as described previously.

2.7. Generation and neutralization of HCVpp

HCV pseudoparticles were produced as previously described (Hsu et al., 2003). Briefly, using calcium phosphate method (Promega), 293T cells were co-transfected with two constructs: pcDNA3.1/Hygro-E1E2 and pNL4-3.Luc.R^-E^- (envelope-deficient HIV-1 proviral genome with a luciferase reporter). Isolates used for HCVpp production included genotype 1a (H77), genotype 1b (UG-P09-779, UG-P10-252 and UG-P12-763) and genotype 2a (JFH1) strains. Supernatant containing HCVpp was collected 72 hours post transfection, sterile filtered through 0.45 µm filter and stored at –80°C. For HCVpp neutralization, $1x10^4$ Hep3B cells were seeded in 96-well tissue culture plate and incubated at 37°C and 5% CO2. The following day, HCVpp were mixed with mAb A6 (100 µg/mL) or anti-E1 mAb IGH526 (10 µg/mL) or anti-E2 mAbs (MRCT10, 1:7, HC84.26, CBH-7 and HC-1AM; 10 µg/mL) or combinations. The mixture was incubated for 1 hour at 37°C before it was added to the Hep3B culture. After 72 hours, intracellular luciferase activity was measured to estimate HCVpp infection (Promega).

2.8. HCVcc production and neutralization

Cell culture-derived HCV (HCVcc) of isolates H77c/JFH1 (genotype 1a), J4/JFH1 (genotype 1b) and J6/JFH1 (Jc1; genotype 2a) was produced as described previously with some modifications (Wakita et al., 2005). Briefly, XbaI linearized HCV plasmids were *in vitro* transcribed (Promega) and the RNA was used for transfection of Huh-7.5RFP-NLS-IPS cells using Lipofectamine 2000 reagent (Invitrogen). Culture supernatant was harvested, sterile filtered and stored at –80°C. For HCVcc neutralization, $1.3x10^4$ Huh-7.5RFP cells were seeded in a 96-well plate. The next day, mAb A6 (100 µg/mL) or MRCT10 (10 µg/mL) was pre-incubated with 50-100 focus forming units (FFUs) of HCVcc at 37°C for 1 hour. The mixture was then added to Huh-7.5RFP cells and incubated for 4 hours followed by washing and incubation for another 48 hours. Infected cells were immunostained with mouse anti-NS5A mAb 9E10 (kindly provided by Dr. Charles Rice, Rockefeller University, USA) and Alexa 647 conjugated goat-anti-mouse IgG (Invitrogen). The number of FFUs were determined using the BD Pathway 435 High Content Bioimager (BD Biosciences). To test the suitability of mAb A6 for immunofluorescence imaging, H77c/JFH1 infected cells were incubated overnight at RT with mAb A6 (1 µg/mL), after which Alexa 647-conjugated goat-anti-human IgG was added for visualization. As a positive control, the same cells were stained with the anti-NS5A mAb 9E10, in combination with Alexa 488-conjugated goat-anti-mouse IgG.

3. Results

3.1. *Amplification of E1E2 from Belgian isolates*

Following E1E2 amplification and agarose gel electrophoresis, bands of approximately 1.7kb, corresponding to full length E1E2 coding genes were cloned into mammalian expression vector and sequenced (data not shown). The names and accession numbers of previously published sequences are listed in Table 1. Multiple sequence alignment and phylogenetic analysis showed that our sequences correspond to 28 new viral isolates (Supplementary Fig. 1) and are of genotype 1b, except for one isolate that is classified as genotype 1a (Fig. 1).

3.2. *Human mAb A6 efficiently binds to recombinant E1E2 proteins*

Hybridoma cell lines were prepared from B lymphocytes of a chronic HCV carrier as described before (Depraetere et al., 2001). Screening of hybridoma culture supernatants identified a clone secreting an HCV E1-specific antibody, designated A6. To evaluate the binding affinity of this antibody to recombinant HCV envelope (E1E2), expression vectors encoding the HCV envelope proteins were used to transfect 293T cells and cell lysates were used in GNA binding ELISA. As shown in Fig. 2A and B, sigmoidal dose response curves were observed when cell lysate containing E1E2 of three genotype 1a (H77, UKN1A20.8 and UKN1A14.38), one genotype 1b (UKN1B12.16) and two genotype 2a (UKN2A1.2 and JFH1) isolates were used. Similar binding experiments were performed using cell lysates containing E1E2 of multiple isolates. These included previously published genotype 1b isolates (J4, UKN1B5.23, P5VD, P5VE and P5VF), the 28 Belgian isolates of genotype 1a and 1b mentioned above as well as isolates of genotype 2b (UKN2B2.8), genotype 3a (S52), genotype 4 (UKN4.11.1), genotype 5 (UKN5.14.4) and genotype 6 (UKN6.5.8). Overall, GNA ELISA binding data showed that mAb A6 antibody efficiently reacts with full length E1E2 envelope glycoproteins of genotypes 1a, 1b and 2a, but not with E1E2 of genotypes 2b, 3a, 4, 5 and 6 (Fig. 2C).

3.3. *Antibody A6 recognizes the region AA 230-239 of E1*

To identify the region critical for mAb A6 binding, we used a custom-made peptide microarray spanning the complete E1E2 sequence. Upon incubation of array chip with mAb A6, reactive spots were observed with peptides spanning amino acids 225-243 of the HCV E1 protein, while no reactivity was seen in any other regions within E1 or E2 (Fig. 3A). The sequences of peptides spanning the full length E1E2 are presented in Supplementary File 1 and peptides with positive reactivity to A6 are highlighted with grey background. To confirm this result, 11 individual peptides spanning the region 222-246 were produced for use in traditional peptide ELISA. As shown in Figure 3B, antibody A6 reacted with peptides spanning the region AA 225-244, thereby confirming the results obtained with the PEPperPRINT microarray assay. Since the peptides used in these assays are not glycosylated, we can assume that the glycosylation of the HCV envelope protein in this region is not essential for mAb binding. To test this hypothesis, we performed peptide-based competition assay. In this assay, ELISA plates pre-coated with recombinant E1E2 protein of H77 isolate were loaded with serial dilutions of individual peptides together with a fixed concentration of mAb A6. This showed the ability of the peptides spanning AA 225-244 to efficiently compete with recombinant E1E2 for binding to mAb A6, which confirms that A6-binding is glycosylation independent (Fig. 3C). The glycosylation-independent reactivity of mAb A6 was confirmed using Western blotting analysis of PNGase F-treated, Endo H-treated and non-treated E1E2 cell lysate, where mAb A6 binds to both glycosylated and deglycosylated E1 (Fig. 4). Overall, our data indicates that the minimal epitope of antibody A6 encompasses AA 230-239 of HCV E1.

3.4. The epitope identified by mAb A6 has a linear conformation

In order to assess whether the A6 epitope has a linear or conformational structure, we performed a denaturation binding assay. Binding of A6 to native and denatured E1E2-containing cell lysate was assessed and compared to the binding activity of antibodies A4 and 1:7, targeting respectively a linear E1-epitope and a conformational epitope within E2. While denaturation of H77 E1E2 only minimally affected binding of antibodies A4 and A6, that of mAb 1:7 was completely abolished (Fig. 5A). Likewise, denaturation of JFH1 E1E2 did not negatively impact A6 binding, while the binding of mAb 1:7 was completely lost (Fig. 5B). The absence of A4 binding to both native and denatured JFH1 E1E2 protein is explained by its non-reactivity towards genotype 2 isolates. Altogether, these data show that the epitope targeted by mAb A6 has a linear, denaturation-resistant structure.

To investigate potential conservation of the A6 epitope among different HCV genotypes, amino acid sequences of E1 for isolates used in the binding assay were aligned using BioEdit and CLC main workbench. As seen from the sequence alignment and the percentage of conservation, certain amino acids in region 230-239, which is critical for mAb A6 binding, are highly conserved especially among genotypes 1a and 1b (Fig. 6). Amino acids cysteine and tryptophan at sites 238 and 239 respectively are conserved in all 7 genotypes. The residues 234N and 236S are also conserved, except in case of genotype 2b where they are replaced by glycine and leucine, respectively. Likewise for 230V, which is only different for genotype 2. Other positions such as 231, 232 and 237 show high conservation among genotypes 1a, 1b and 2a compared to genotypes 2b, 3a, 4, 5 and 6.

3.5. Antibody A6 does not neutralize HCVpp and HCVcc

Since mAb A6 efficiently binds to E1E2, we next wanted to investigate its neutralizing activity. Therefore, HCVpp from genotypes 1a (H77 isolate), 1b (UG-P09-779, UG-P10-252 and UG-P12-763) and 2a (JFH1 isolate) were produced and used in neutralization assays. Incubation of mAb A6 with HCVpp before addition to Hep3B cell cultures did not protect the target cells from infection, even at high mAb concentration (100 µg/mL) (Fig. 7A-E). In parallel, 10 µg/mL of the AP33-derived humanized mAb MRCT10 was included as positive control and completely prevented HCVpp infection of all tested genotypes. These results indicate that mAb A6 does not exhibit any neutralizing activity in the HCVpp system, despite its capacity to efficiently bind the viral envelope.

Subsequently we also tested the neutralizing capacity of mAb A6 in the context of the HCV cell culture (HCVcc) system. Three universally used chimeric viruses H77c/JFH1, J4/JFH1 and Jc1 (J6/JFH1), incorporating the envelope proteins of isolates H77c (genotype 1a), J4 (genotype 1b) and J6 (genotype 2a) respectively, were pre-incubated with A6 or MRCT10, after which the mixture was transferred to Huh7.5 hepatoma cells. Enumeration of the number of infected foci 2 days later showed that mAb A6 was unable to protect Huh7.5 cells from HCVcc challenge, while MRCT10 did (Fig. 7F-H). Altogether, our HCVpp and HCVcc data indicate that mAb A6 has no neutralizing activity. However, staining of H77c/JFH1 infected Huh7.5RFP cells with mAb A6 enabled the visualization of infected cells which indicates the applicability of mAb A6 for use in immunofluorescence (Fig. 8).

3.6. *mAb A6 does not interfere with neutralizing antibodies*

A broadly neutralizing determinant encompassing residues 412-423 of E2 has previously been shown to be susceptible to antibody interference (Kachko et al., 2015; Zhang et al., 2009), although this is controversial (Tarr et al., 2012). In this regard, we examined if antibody A6 could interfere with the binding and/or protective activity of other known neutralizing antibodies. Murine mAb AP33 is one of the most extensively studied antibodies that target a linear epitope within HCV E2 protein and exhibits broad neutralizing activity both *in vitro* and *in vivo* (Desombere et al., 2016; Owsianka et al., 2005). In a first setup, full length E1E2 protein of isolate H77 was incubated with serial dilutions of mAb A6 before addition of biotinylated AP33. Subsequent addition of HRP-conjugated streptavidin enabled us to assess the amount of biotinylated antibody that still was able to bind to the envelope proteins. As can be seen in Figure 9A, mAb A6 did not interfere with the binding the biotinylated AP33 whereas the humanized version of AP33, mAb MRCT10, inhibited the subsequent binding of biotinylated AP33 in a dose-dependent manner. To confirm the non-interfering nature of A6, HCVpp neutralization was performed using H77 and JFH1 isolates in the presence of mAbs A6, MRCT10, IGH526, 1:7, HC84.26, CBH-7 and HC-1AM alone or in combination. Measurement of luciferase activity showed that MRCT10 efficiently inhibited HCVpp infection of both genotypes, while the co-incubation of mAb A6 did not interfere with this activity (Fig. 9B and C). Similarly, mAb A6 did not negatively impact the neutralizing activity of mAbs IGH526, 1:7, HC84.26, CBH-7 and HC-1AM. These mAbs have been previously shown to have cross-neutralizing activity against HCVpp and HCVcc (Johansson et al., 2007; Keck et al., 2004a; Keck et al., 2012; Wang et al., 2011b). Based on our ELISA and HCVpp competition experiments we can conclude that our anti-E1 mAb A6 does not interfere with the neutralizing activity of known anti-E2 mAbs.

4. Discussion

In the current study, we describe the development and characterization of a novel human monoclonal antibody (designated A6) isolated by hybridoma technology from the PBMCs of a patient chronically infected with HCV genotype 1b. Epitope mapping identified the region encompassing AA 230-239, located at the N-terminal region of E1, as the critical region for binding. Previously, two other regions within E1 have been described that elicit E1-specific antibodies. The first is the N-terminal region recognized by human mAb H-111 (AA 192-202) (Keck et al., 2004b) and murine mAb A4 (AA 197-207) (Dubuisson et al., 1994), which lies upstream of the epitope recognized by A6. The second antigenic region within E1 encompasses AA 313-327, which is located at the C-terminal region and is recognized by cross-neutralizing mAbs such as IGH505 and IGH526 (Meunier et al., 2008). mAb H-111 was isolated from a patient chronically infected with HCV of genotype 1b and showed high binding activity to E1 protein from genotypes 1a, 1b, 2b. It bound only moderately to HCV of genotype 3a, and no binding was observed to genotype 2a and 4a. It also exhibited some neutralizing activity towards HCV-like particles and HCVpp, but complete prevention of infection was not achieved (Dreux et al., 2006; Keck et al., 2004b). To evaluate the binding activity of mAb A6 , we used full length E1E2 protein that was expressed in mammalian cells. New isolates from Belgian patients, genotyped as 1a and 1b, have been used together with well-known isolates covering genotypes 1 to 6. Active binding of mAb A6 to all isolates of genotypes 1a (4 isolates), 1b (33 isolates) and 2a (2 isolates) was observed, while no binding activity was seen with the other genotypes (2b, 3a, 4, 5 and 6; one isolate for each genotype). This resembles the mAb H-111 in binding to genotypes 1a and 1b (Keck et al., 2004b). The major difference between the mAb H-111 study and A6 is that we used full length E1E2 proteins instead of E1. Altogether these data confirm that the activity of antibodies raised against the N-terminal region of E1 is genotype dependent.

Although the sequence of the HCV envelope exhibits great variability across genotypes, conserved glycosylation sites in E1, including the N-terminal region, and E2 have been identified and shown to play a role in evasion from host immunity (Dubuisson et al., 1994; Goffard and Dubuisson, 2003; Helle et al., 2011; Helle et al., 2010). Since the recombinant E1E2 proteins used in our binding ELISA were expressed in mammalian cells, their proper glycosylation and post-translational modification may be expected. In contrast, synthetic peptides used in epitope mapping are non-glycosylated. For this reason, we have also examined the effect of these peptides in a competition assay together with recombinant E1E2 protein. The strong inhibition of binding of mAb A6 to E1E2 glycoprotein upon addition of synthetic peptides indicates that this binding is not relying on the glycosylation of E1. This was corroborated by Western blot analysis, which showed positive reactivity of mAb A6 to both glycosylated and (PNGase/Endo H) deglycosylated E1 (Dubuisson et al., 2000). In addition, the envelope of HCV contains multiple intramolecular disulfide bonds which are essential for proper folding and stabilizing (Krey et al., 2010). Incubation of recombinant envelope glycoprotein with SDS and DTT at high temperature destroys these disulfide bonds and disrupts the secondary structure of the protein. This approach has been used to differentiate between antibodies targeting linear versus conformation-dependent epitopes (Keck et al., 2012; Tarr et al., 2006). mAb A6 efficiently recognized the envelope of both native and denatured E1E2 proteins and a similar result was obtained for mAb A4 that previously shown to target a linear epitope within E1. In parallel, mAb 1:7 that targets a conformational epitope within E2 completely lost its binding upon denaturation of the envelope proteins, confirming previous findings (Allander et al., 2000; Johansson et al., 2007). Altogether, our results indicate that the mAb A6 targets a linear epitope within the N-terminus of the E1 protein. Multiple sequence alignment of the E1 protein using all isolates used in the current study in addition to previously published sequences revealed the highly conserved nature of residues 230, 234, 236, 238 and 239 within the A6 epitope among different genotypes. However, the change of polar amino acids at residues 234 and 236 to more hydrophobic ones may be the underlying cause of the absence of reactivity of mAb A6 towards genotype 2b isolates. On the other hand, residues at sites 231, 232 and 237 are highly conserved among isolates of genotypes 1a, 1b and 2a compared to the high variation observed in other genotypes like 2b, 3a, 4, 5 and 6. Altogether this may explain the efficient and specific binding of mAb A6 with the E1 envelope protein of isolates of genotypes 1a, 1b and 2a. Similar sequence conservation has been shown upon alignment of the C-terminal region, a region that is identified by cross-neutralizing antibodies (Bukh et al., 1993; Meunier et al., 2008).

Using the HCVpp system, mAb A6 could not inhibit the entry of pseudoparticles of genotypes 1a, 1b and 2a, while complete protection was achieved by mAb MRCT10 - the humanized version of the cross-neutralizing mAb AP33 (Owsianka et al., 2001; Pantua et al., 2013). Previous studies showed low neutralizing activity of the human mAb H-111 while the neutralizing potential of mAb A4 has not been reported (Dubuisson et al., 1994; Keck et al., 2004b). Nevertheless, the non-neutralizing function of antibodies raised against the N- terminal region cannot be generalized to all E1-specific mAbs since the two mAbs, IGH505 and IGH526, that target the region AA 313-327 showed cross-neutralizing capacity when tested in the HCVpp and HCVcc systems (Meunier et al., 2008). Since HCVpp are produced in 293T cells, a non-hepatic cell line, its lipoprotein profile may differ from the that of genuine HCV virions produced in infected patients and from HCVcc produced in hepatic cell lines. In order to confirm the results obtained with the HCVpp system, we performed similar neutralization experiments in the HCVcc system. Despite the use of high antibody concentration mAb A6 was also unable to protect Huh7.5 cells from infection.

Some studies have reported that antibodies raised against certain epitopes within E2 interfere with the neutralizing activity of other antibodies. For example, antibodies targeting epitope II (AA 434-446) within E2 protein have been shown to interfere with the neutralizing activity of other mAbs targeting epitope I (AA 412-424) (Kachko et al., 2015; Zhang et al., 2007; Zhang et al., 2009) although others contradicted this finding (Tarr et al., 2012). No data is available about the potential interfering activity of E1 antibodies. We therefore examined whether A6 interfered with the activity of anti-E1 antibody IGH526 and anti-E2 mAbs MRCT10 (epitope I-specific), 1:7 (AA 523-535; domain B-specific), HC84.26 (epitope II-specific), CBH-7 (domain C-specific) and HC-1AM (domain B-specific) and found out that it did not. A recent study reported that antibodies against non-neutralizing epitopes within E1 and E2 can be involved in antibody-dependent cytotoxic responses by natural killer cells (Long et al., 2017). This new finding suggests that non-neutralizing, non-interfering antibodies like A6 may still play a role in the immune control of HCV. More in depth exploration of this mechanism is warranted.

In conclusion, we report that the region spanning AA 230-239 at the N-terminal region of E1 is immunogenic and can elicit non-neutralizing Abs during a chronic HCV infection cross-reactive to genotypes 1a, 1b and 2a. mAb A6 can be considered as one more tool that could help to elucidate the structure of the viral envelope, an important step in the development of an effective prophylactic HCV vaccine.

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Figure legends

Fig. 1. Phylogenetic tree for new E1E2 isolates. Following isolation and sequencing of full length E1E2 from Belgian patients infected with HCV, sequences were multiple aligned together with previously published sequences retrieved from the HCV database. Neighbor joining phylogenetic tree was constructed using CLC main workbench based on the whole E1E2 amino acid sequence. Analysis shows that all Belgian isolates belong to genotype 1b, except for one isolate genotyped as 1a. The 28 Belgian genotype 1a and 1b isolates are highlighted in red. Bootstrap values are indicated.

Fig. 2. Binding of mAb A6 to recombinant E1E2. Constructs encoding the full length E1E2 of HCV genotypes 1-6 were used for expression in 293T cells. GNA-coated ELISA plates were loaded with cell lysate containing E1E2. (A and B) Binding of serially diluted mAb A6 was assessed using ELISA plates coated with E1E2 of three genotype 1a isolates (H77, UKN1A20.8, UKN1A14.38), one genotype 1b (UKN1B12.16) and two genotype 2a isolates (UKN2A1.2 and JFH1). (C) mAb A6 binding to the envelope proteins of multiple other isolates of different genotypes was evaluated at fixed antibody concentration (10 µg/mL). Lysate of non-transfected 293T cells was included as a negative control. All conditions were performed in duplicate and error bars represent the standard deviation.

Fig. 3. Epitope mapping of mAb A6. (A) To identify the epitope within E1E2 recognized by mAb A6, a custom-made chip (PEPperPRINT) with printed overlapping peptides spanning the complete E1E2-sequence was used. After incubation with mAb A6 and an Alexa647-conjugated goat anti-human IgG, spots corresponding to 5 different E1-specific peptides appeared (highlighted with black square). The reactive peptides were AA 225-239, AA 226-240, AA 227-241, AA 228-242 and AA 229-243. The positive spots in the outer rim of the chip are controls that are used for localization purposes only. (B) Standard peptide ELISA was performed using synthetic peptides spanning the region AA 222-246 (25 µg/mL). mAb A6 was used at 10 µg/mL and binding activity is shown by OD values measured at 450nm. (C) A peptide competition assay was performed on ELISA plates coated with recombinant E1E2 (H77 isolate), using a fixed concentration of mAb A6 (20 µg/mL) in the presence of serial dilutions of the indicated synthetic peptides. Binding of mAb A6 is denoted as % of maximal binding in the absence of synthetic peptide. The amino acid sequence of the peptides covering the region AA 222-246 used in (A-C) is indicated.

Fig. 4. Reactivity of mAb A6 to glycosylated and deglycosylated E1E2 protein. Lysate of 293T cells expressing H77 E1E2 was treated with PNGase F or Endo H. Following 12% SDS-PAGE, proteins were transferred to a nitrocellulose membrane and revealed using mAb A6 as primary antibody. Positive bands were visible at a molecular weight around 20-22 kDa in case of PNGase F and Endo H-treated cell lysate (lane 3 and 4), which correspond to deglycosylated E1, while the untreated lysate showed reactive bands at around 25-32 kDa (lane 2). The position and molecular weights of protein standard are presented in kDa (lane M). The deglycosylated E1 protein is indicated by an asterisk.

Fig. 5. Antibody A6 targets a linear epitope. To investigate if the region targeted by mAb A6 is conformation sensitive or not, cell lysate containing E1E2 of isolate H77 (genotype 1a) (A) or JFH1 (genotype 2a) (B) was denatured by incubation with SDS and DTT at 56°C. Native and denatured cell lysate were coated on ELISA plates and binding of mAb A6 (5 µg/mL) was assessed. In parallel, murine mAb A4 and human mAb 1:7 were included as controls for linear and conformational epitopes respectively. All conditions were performed in duplicate and results are shown as the mean +/- standard deviation.

Fig. 6. Multiple alignment of the region corresponding to AA 192-250 and AA 310-330 of HCV E1. The E1 sequence of all HCV isolates used in the current study was retrieved from GenBank and the HCV database and multiple aligned using BioEdit software. The epitopes targeted by antibodies H-111, A4, A6, IGH505 and IGH526 are indicated using colored squares. The dots indicate conservation of the residue relative to the reference prototype H77 (genotype 1a). The lower part of the alignment shows the consensus sequence and the percentage of overall conservation calculated using CLC Main Workbench.

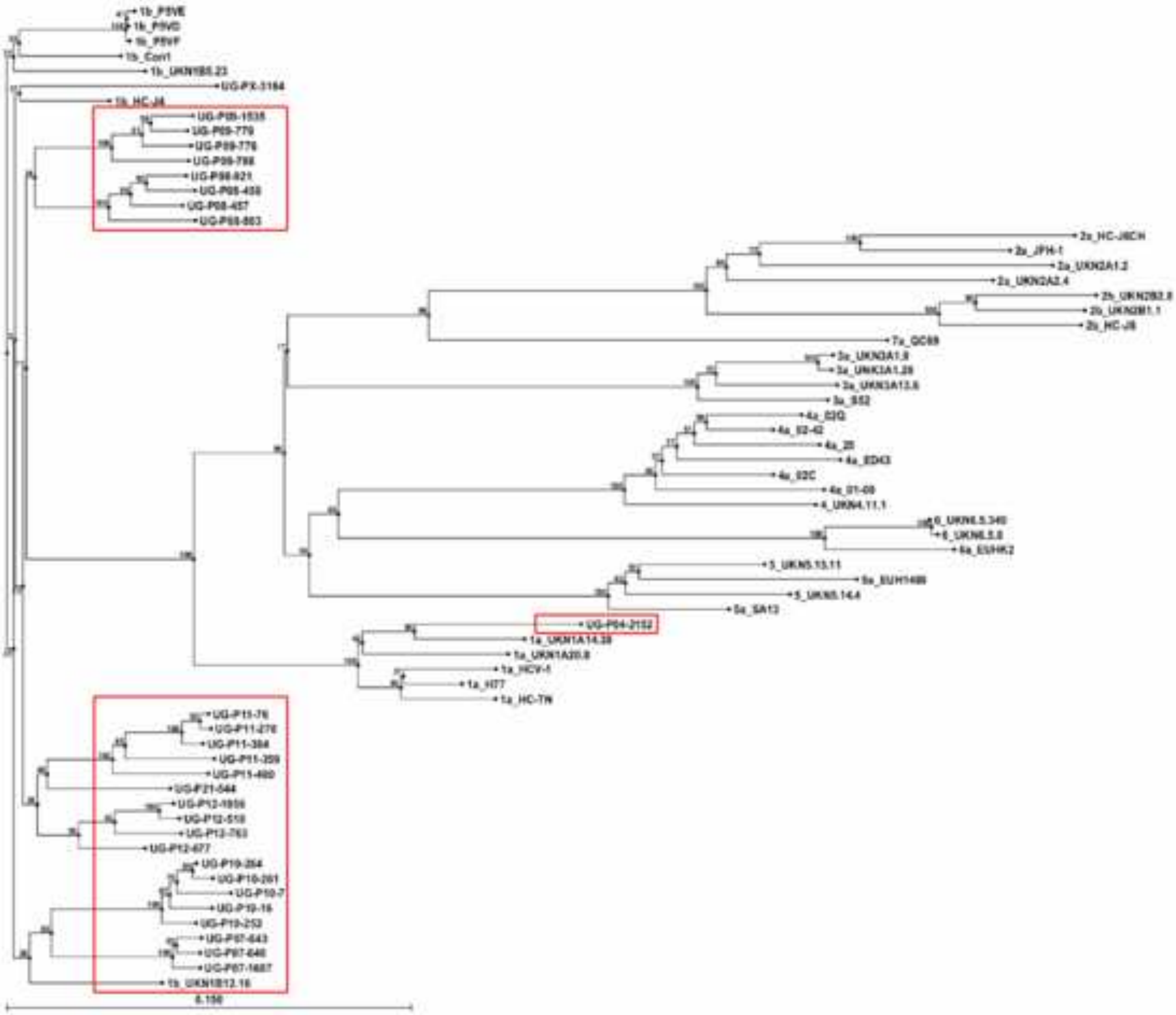
Fig. 7. HCVpp and HCVcc neutralization. In order to verify the neutralizing capacity of mAb A6, HCVpp expressing E1E2 of H77 (genotype 1a) (A), UG-P09-779 (genotype 1b) (B), UG-P10-252 (genotype 1b) (C), UG-P12-763 (genotype 1b) (D) and JFH1 (genotype 2a) (E) were incubated for 1 hour at 37°C with mAb A6 (100 µg/mL) or with mAb MRCT10 (10 µg/mL) before addition to Hep3B cells. After 72 hours, HCVpp infection was assessed by quantifying the luciferase reporter gene expression and the results were normalized to untreated controls (Ctrl). Chimeric HCVcc of genotypes 1a/2a (F), 1b/2a (G) and 2a/2a (H) were pre-incubated with mAb A6 at a concentration of 100 µg/mL or with 10 µg/mL of mAb MRCT10 for 1 hour at 37 °C before exposure to Huh7.5RFP cells. The number of HCV-infected

foci was counted using NS5A-specific antibody staining. The infection was normalized to that in cultures that were exposed to virus only (Ctrl). All conditions were performed in triplicate and results are shown as the mean +/- standard deviation.

Fig. 8. mAb A6-based visualization of infected Huh7.5RFP cells using immunofluorescence. H77c/JFH1 infected Huh7.5RFP cells (upper panel) and non-infected cells (-ve; lower panel) were incubated overnight with mAb A6 (middle column), after which Alexa 647-conjugated goat-anti-human IgG was added. As a positive control, the same cells were stained with anti-NS5A mAb 9E10 in combination with Alexa 488 conjugated goat-anti-mouse IgG (right column). DAPI was used as counterstain (left column).

Fig. 9. mAb A6 does not interfere with neutralizing antibodies. (A) Competition ELISA was performed using biotinylated AP33 and E1E2 of isolate H77 as a coating antigen. Serial dilutions of mAb A6 were used starting at 30 µg/ml and the binding of biotinylated AP33 was measured using HRP-conjugated streptavidin. As a positive control for inhibition, serial dilutions of MRCT10 were used in parallel. To investigate the interference with the neutralizing potential of anti-E2 antibodies, HCVpp from genotype 1a (H77) (B) and 2a (JFH1) (C) were used. Co-incubation of mAb A6 (100 µg/mL) and one of anti-E1 or anti-E2 mAbs (IGH526, MRCT10, 1:7, HC84.26, CBH-7, HC-1AM; 10 µg/mL) and HCVpp was done one hour before addition to Hep3B cells. The luciferase activity was determined and the percentage of neutralization was calculated relative to infection in the absence of antibody (Ctrl). All conditions were performed in duplicate for ELISA and 5 replicates for HCVpp inhibition. Results are shown as the mean +/- standard deviation.

Figure 1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1b_P5VF
1b_Con1
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1b_HC-J4
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UG-P09-779
UG-P09-776
UG-P09-788
UG-P08-821
UG-P08-438
UG-P08-457
UG-P08-883
2a_HC-J6CH
2a_JFH-1
2a_UKN2A1.2
2a_UKN2A2.4
2b_UKN2B2.8
2b_UKN2B1.1
2b_HC-J8
7a_QC69
3a_UKN3A1.9
3a_UKN3A1.28
3a_UKN3A13.6
3a_S52
4a_03Q
4a_02-42
4a_25
4a_ED43
4a_02C
4a_01-09
4_UKN4.11.1
6_UKN6.5.340
6_UKN6.5.8
6a_EUHK2
5_UKN5.15.11
5a_EUH1480
5_UKN5.14.4
5a_SA13
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1a_HCV-1
1a_H77
1a_HC-TN
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UG-P11-276
UG-P11-384
UG-P11-359
UG-P11-460
UG-P21-544
UG-P12-1956
UG-P12-518
UG-P12-763
UG-P12-677
UG-P10-264
UG-P10-261
UG-P10-7
UG-P10-16
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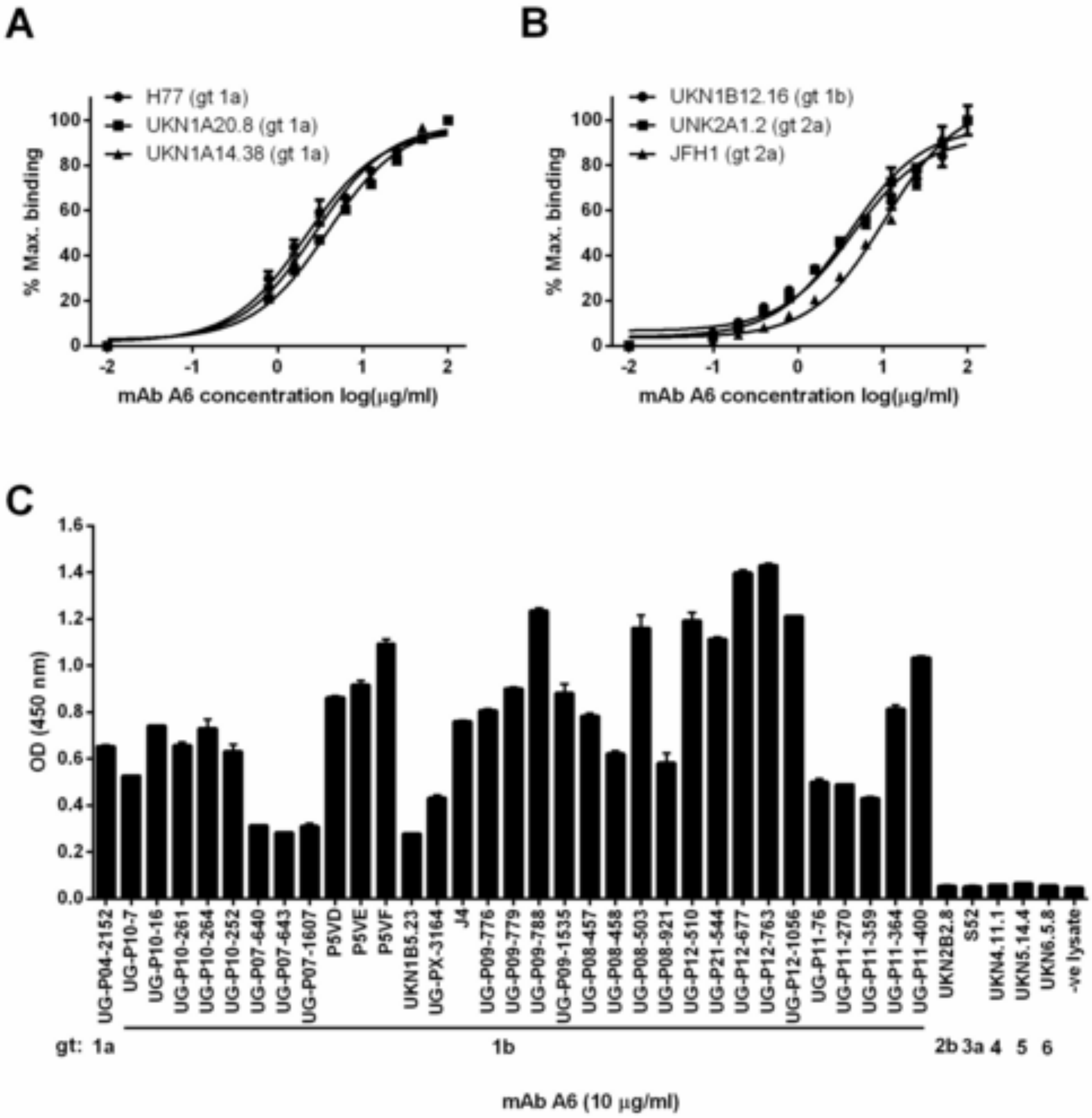
Figure 2


A
H77 (gt 1a)
UKN1A20.8 (gt 1a)
UKN1A14.38 (gt 1a)
% Max. binding
0
20
40
60
80
100
-2
-1
0
1
2
mAb A6 concentration log(µg/ml)
B
UKN1B12.16 (gt 1b)
UNK2A1.2 (gt 2a)
JFH1 (gt 2a)
% Max. binding
0
20
40
60
80
100
-2
-1
0
1
2
mAb A6 concentration log(µg/ml)
C
OD (450 nm)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
UG-P04-2152
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P5VD
P5VE
P5VF
UKN1B5.23
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J4
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UG-P08-503
UG-P08-921
UG-P12-510
UG-P21-544
UG-P12-677
UG-P12-763
UG-P12-1056
UG-P11-76
UG-P11-270
UG-P11-359
UG-P11-364
UG-P11-400
UKN2B2.8
S52
UKN4.11.1
UKN5.14.4
UKN6.5.8
-ve lysate
gt:
1a
1b
2b
3a
4
5
6
mAb A6 (10 µg/ml)

Figure 3

Figure 3

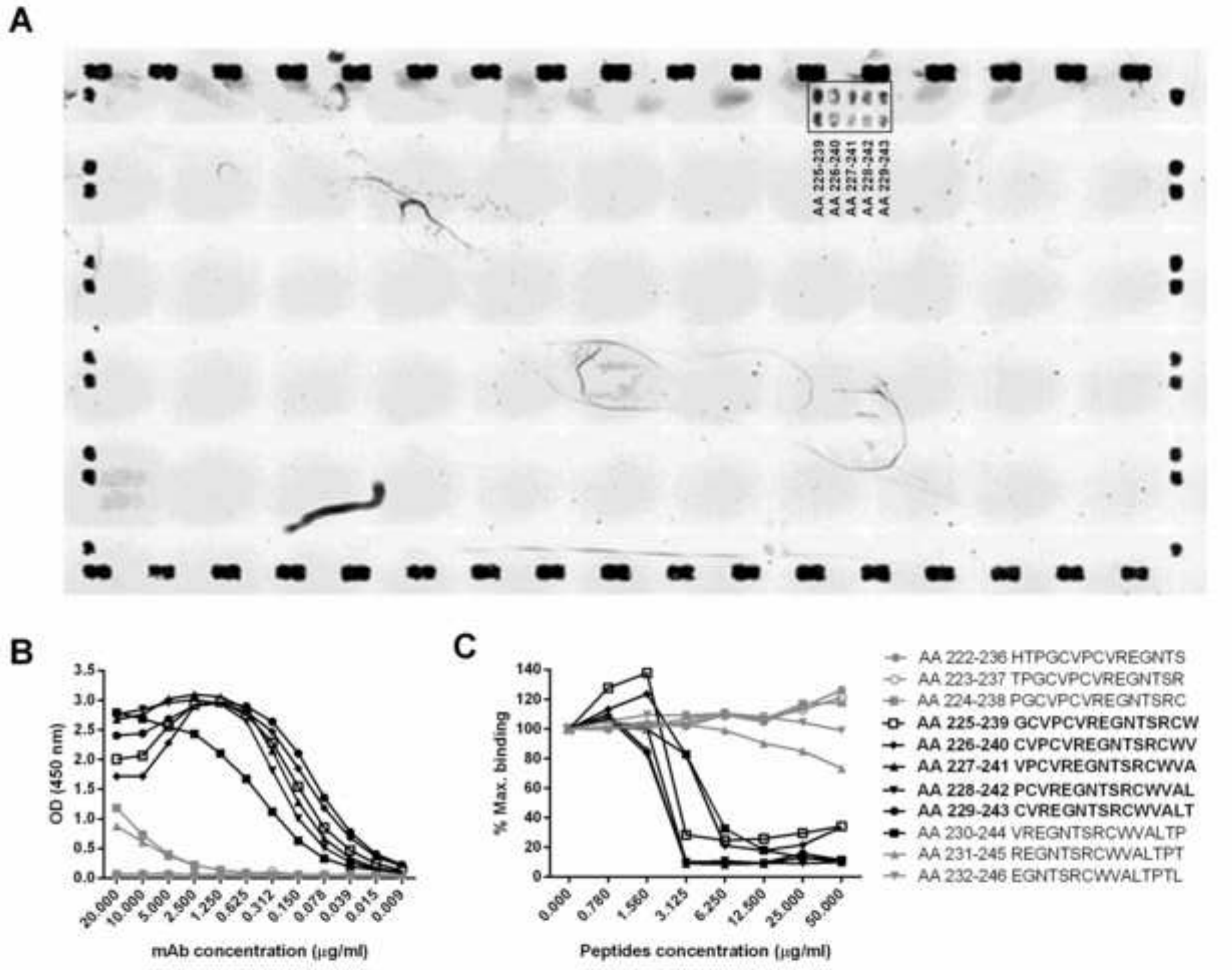

Figure 4
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Figure 4

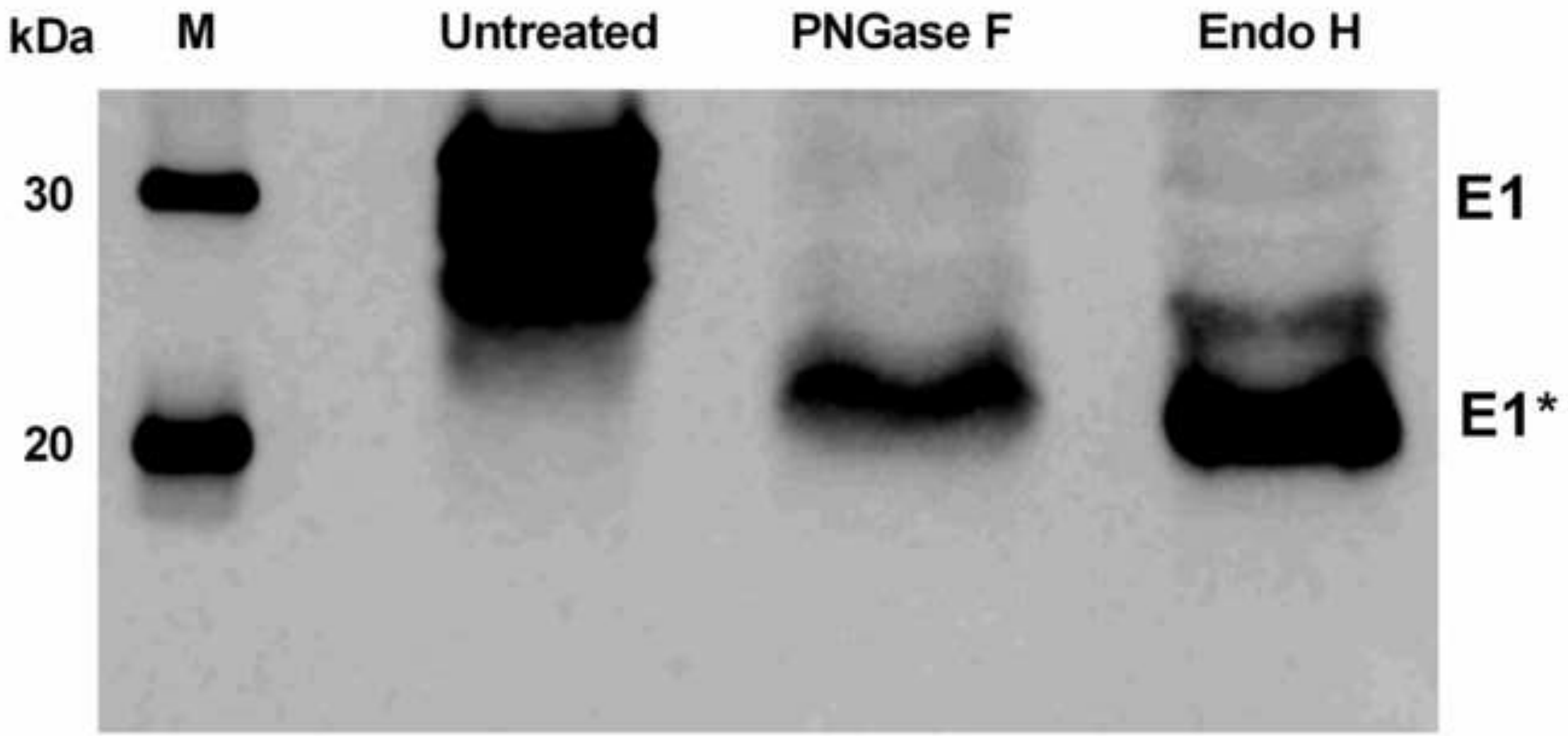

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Figure 5

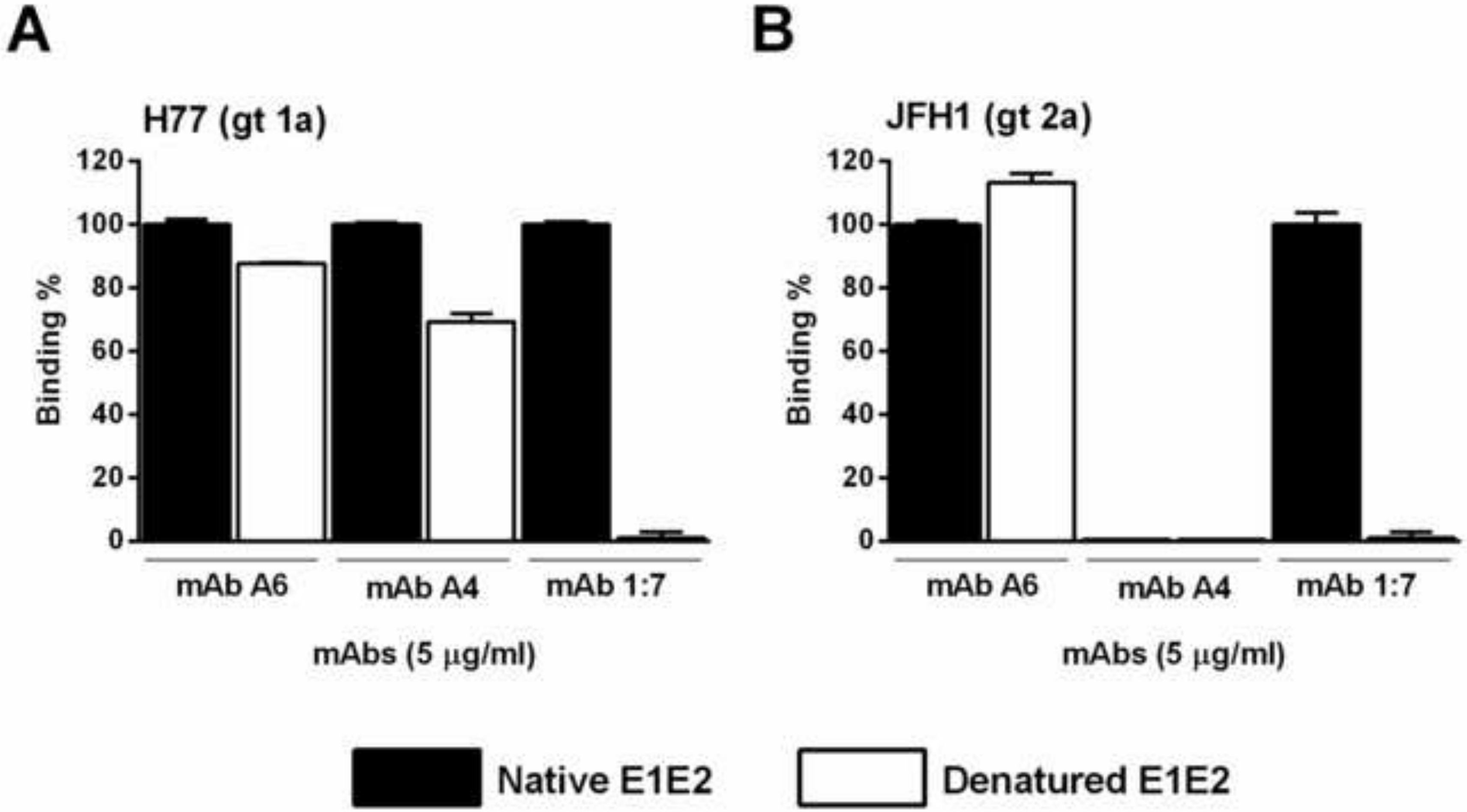

Figure 6

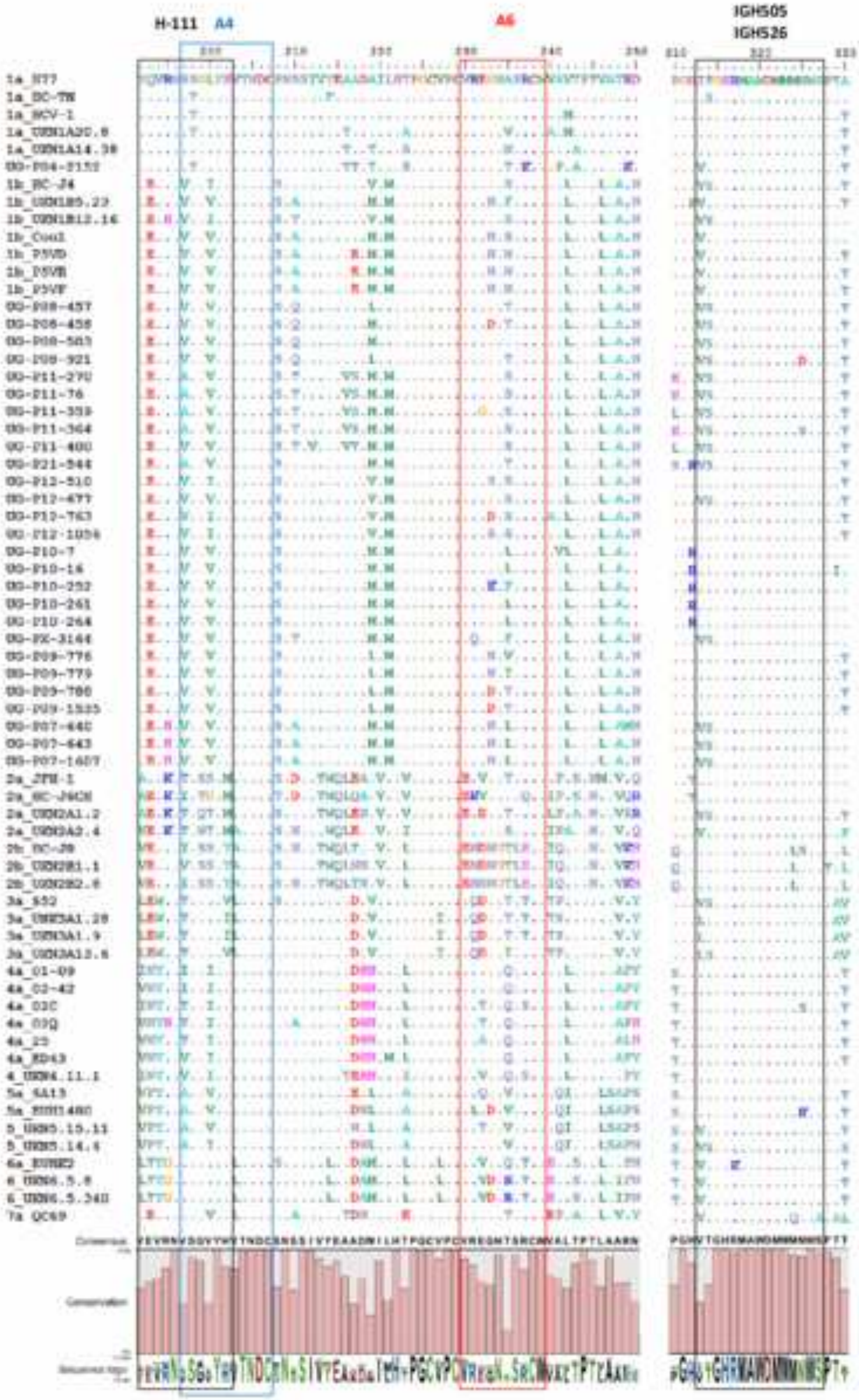
H-111
A4
A6
IGH505
IGH526
Consensus
Conservation
Sequence logo

Figure 7

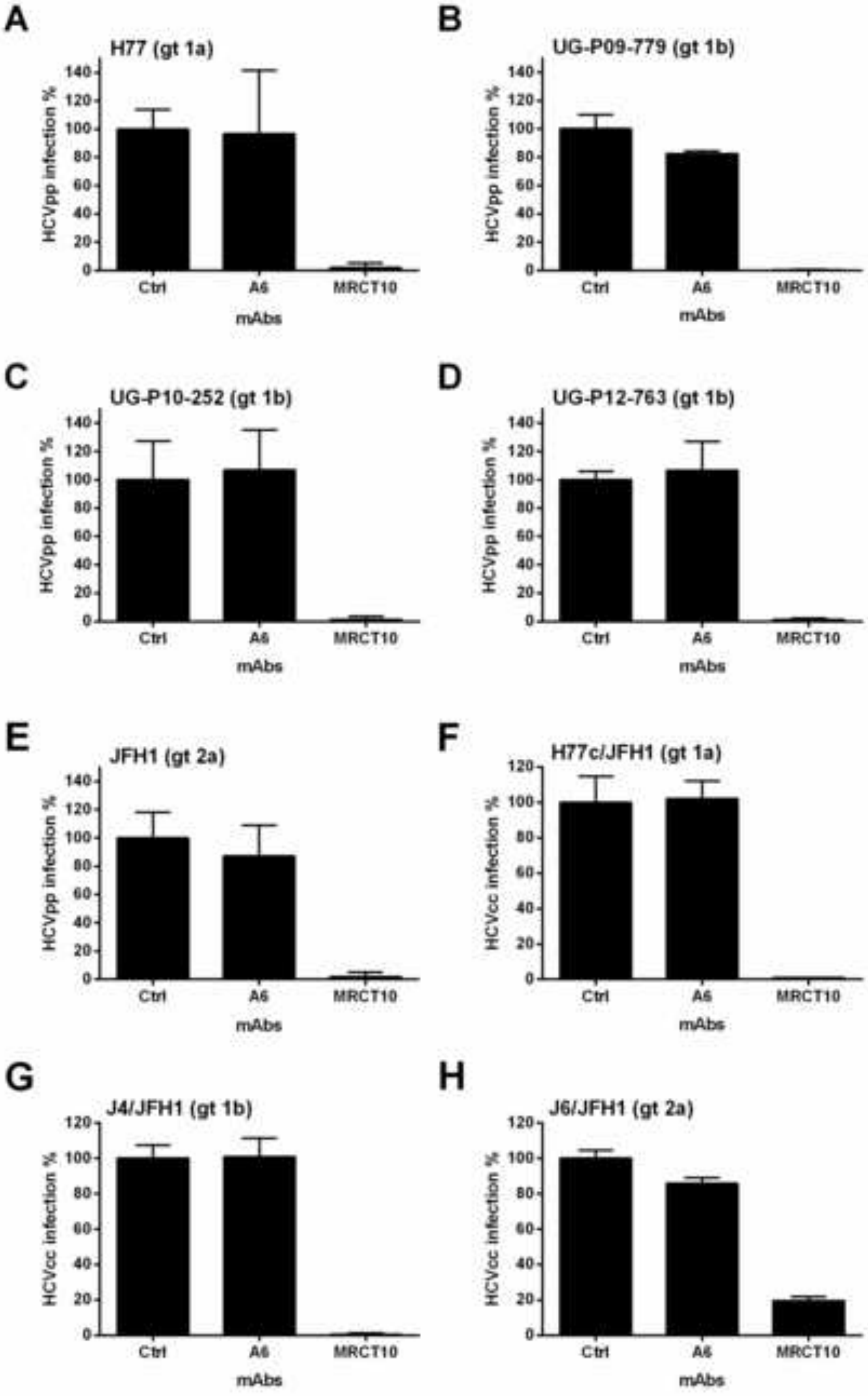
A
H77 (gt 1a)
B
UG-P09-779 (gt 1b)
C
UG-P10-252 (gt 1b)
D
UG-P12-763 (gt 1b)
E
JFH1 (gt 2a)
F
H77c/JFH1 (gt 1a)
G
J4/JFH1 (gt 1b)
H
J6/JFH1 (gt 2a)
HCVpp infection %
HCVcc infection %
0
20
40
60
80
100
120
140
Ctrl
A6
MRCT10
mAbs

Figure 8

Figure 8

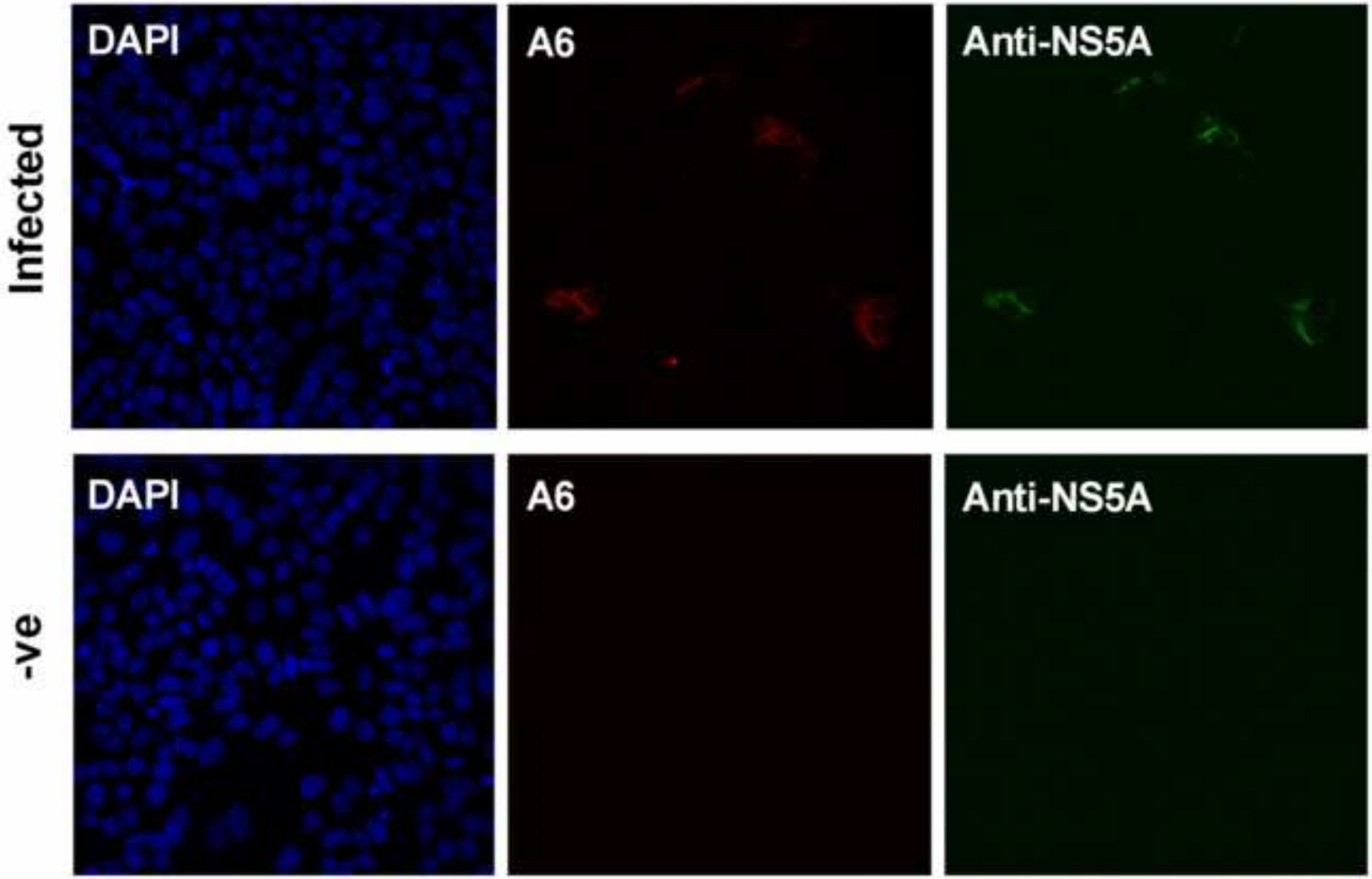

Figure 9

Figure 9

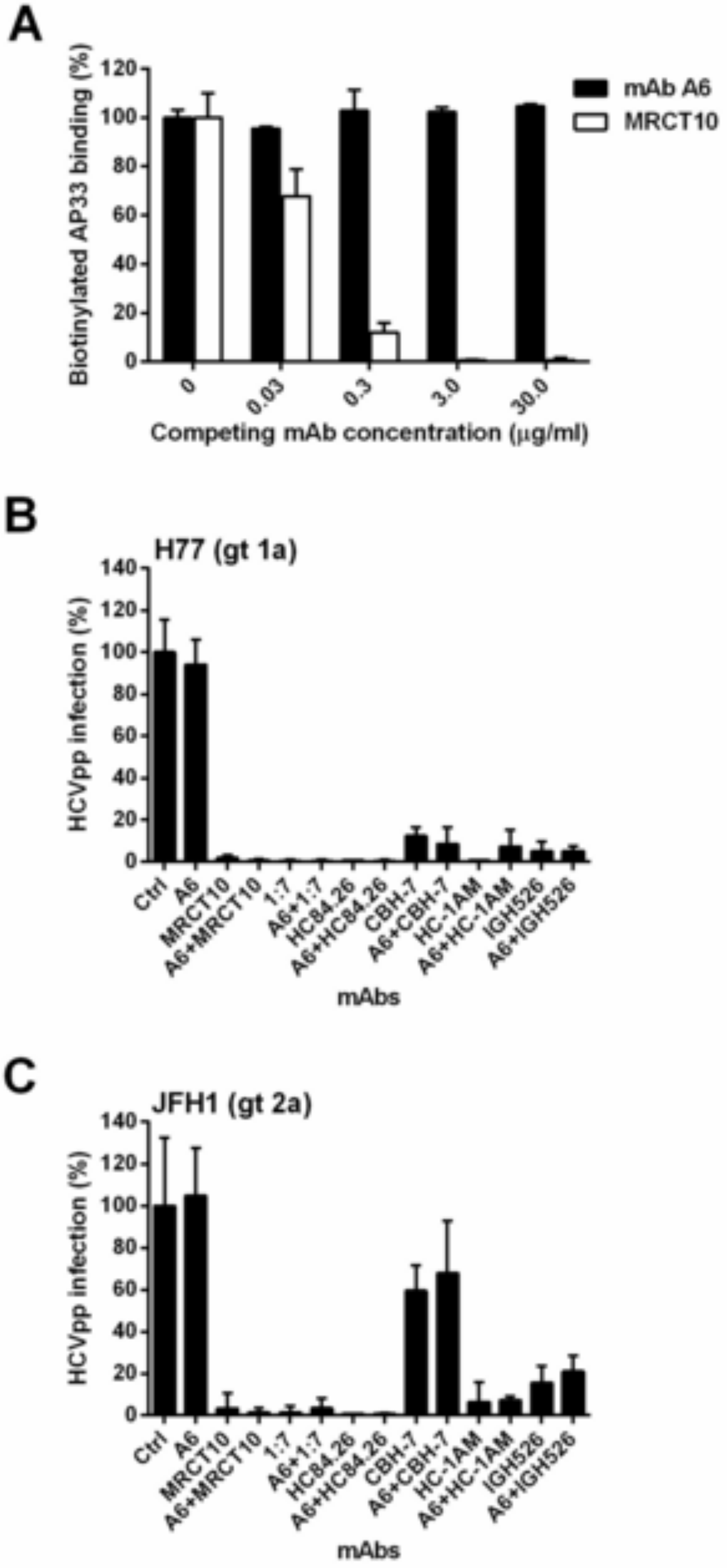

Table 1: Names, genotypes and accession numbers for the E1E2 isolates used in ELISA.

Isolate ID	Genotype	Accession number
H77	1a	AF011751
UKN1A20.8	1a	EU155192
UKN1A14.38	1a	AY734971
J4	1b	AF054250
UKN1B5.23	1b	AY734976
UKN1B12.16	1b	AY734974
P5VD	1b	(Fafi-Kremer et al., 2010)
P5VE	1b	(Fafi-Kremer et al., 2010)
P5VF	1b	(Fafi-Kremer et al., 2010)
UG-P08-503	1b	(Desombere et al., 2016)
UG-P09-779	1b	(Desombere et al., 2016)
UG-P10-252	1b	(Desombere et al., 2016)
UG-P12-763	1b	(Desombere et al., 2016)
JFH1	2a	AB047639
UKN2A1.2	2a	AY734977
UKN2B2.8	2b	AY734983
S52	3a	GU814263
UKN4.11.1	4	AY734986
UKN5.14.4	5	AY785283
UKN6.5.8	6	EF427671

Supplementary Figure 1

Supplementary File 1

Agreements additional co-author